Studies on the mechanism of luteinizing hormone-induced expression of prostaglandin E_2 receptor EP4b essential for ovulation of the medaka

(メダカ排卵に必須なプロスタグランジン E_2 受容体 EP4b の LH による発現誘導機構に関する研究)

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Akane Hagiwara

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CONTENTS

ABBREVIATIONS

DHP: 17α, 20β-dihydroxy-4-pregnen-3-one

20β-S: 17α, 20β, 21-trihydroxy-4-pregnen-3-on

Ab: antibody

AC: adenylate cyclase

ANOVA: analysis of variance

AP: alkaline phophatase

AS: antisense strand

BM: basement membrane

bp: base pair

cAMP: cyclic adenosine 3´, 5´-monophosphate

cDNA: complementary deoxyribonucleic acid

ChIP: chromatin immunoprecipitation

CHO: chinese hamster ovary

COC: cumulus-oocyte complex

COX: cyclooxygenase

CREB: cAMP response element binding protein

cytoplasmic actin: actb

DIG: digoxigenin

DMEM: Dulbecco's modified Eagle's medium

ECM: extracellular matrix

EGF: epidermal growth factor

EM: egg membrane

EP: prostaglandin E_2 receptor

FBS: fetal bovine serum

FP: prostaglandin $F_{2\alpha}$ receptor

FSH: follicle stimulating hormone

FSK: forskolin

GC: granulosa cell

GE: germinal epithelium

GVBD: germinal vesicle breakdown

h: hour(s)

HEK: human embryonic kidney

LH: luteinizing hormone

IgG: immunoglobulin G

kD: kilo dalton

MIH: maturation-inducing hormone

MIS: maturation-inducing steroid

MMP: matrix metalloproteinase

MMTV: mouse mammary tumor virus

MPF: maturation- promoting factor

mPR: membrane progestin receptor

NCBI: National Center for Biotehnology Information

nPR: nuclear progestin receptor

OC: oocyte

Ov: ovulation

P4: progesterone

Pai-1: plasminogen activator inhibitor-1

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PG: prostaglandins

PKA: protein kinase A

PRE: progesterone response element

PVDF: Poly Vinylidene DiFluoride

RNA: ribonucleic acid

rLH: recombinant LH

RT: reverse transcription

SDS-PAGE: sodium dodecyl sulphate-poly acrylamide gel electrophoresis

SEM: standard error of the mean

SS: sense strand

TC: theca cell

TLS: trilostane

uPA: urokinase-type plasminogen activator

GENERAL INTRODUCTION

Ovulation is a precisely timed process by which one or more viable oocytes are discharged from mature follicles in the ovary. This important biological process occurs in all vertebrate ovaries. Successful ovulation requires stimulation by luteinizing hormone (LH) derived from the pituitary gland [Richards and Kersey, 1979; Schwartz, 1974]. During prophase of the first meiotic division in mammalian ovaries, the oocyte is enclosed by somatic cells, cumulus cells and granulosa cells (GCs) [Borum, 1961]. Cumulus cells surround the oocyte to form a unique structure known as the cumulus-oocyte complex (COC). The compartment containing the COC and follicle cavity is further surrounded by the follicle wall containing mural GCs and theca cells (TCs). LH activates multiple signaling pathways involving cyclic adenosine 3´, 5´-monophosphate (cAMP)/protein kinase A (PKA), ERK1/2, epidermal growth factor (EGF), etc. As a result, the intercellular space among cumulus cells expands (COC expansion) [Camaioni et al., 1993; Fan et al., 2009], and the oocyte is eventually released. A critical role for prostaglandin (PG) during COC expansion has been demonstrated [Davis et al., 1999].

PGs are hormone-like substances required for many vital physiological processes and were initially discovered as substances associated with sites of inflammation [Floman and Zor., 1976]. Subsequently, PGs, particularly PGE_2 and $PGF_{2\alpha}$, were demonstrated to play a role in female reproduction [Hizaki et al., 1999; Labhsetwar, 1972]. The actions of PGE_2 and $PGF_{2\alpha}$ are mediated by their specific receptors, EP or FP, in vertebrates, including fish [Jalabert and Szöllösi, 1975] and mice [Neal et al., 1975]. Previous studies have established LH-induced PGE_2 production [Bauminger and Lindner, 1975]. Further, studies using mice lacking the EP2 receptor revealed that the PGE_2/EP2 axis is involved in COC expansion [Hizaki et al., 1999]. The luteolytic effect

of $PGF_{2\alpha}$ in mammalian species has also been documented [Labhsetwar, 1972].

PG synthesis is initiated by the release of arachidonic acid from the plasma membrane by phospholipase A_2. Arachidonic acid is then converted to PGH_2 by an enzymatic two-step reaction involving cyclooxygenase (COX) -1 or -2. PGH_2 is subsequently converted to one of the PGs (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, or TXA_2) by specific PG synthases [Funk, 2001]. Previous studies have established that COX enzymes play an important role in PG synthesis. COX-1 is reported to be a constitutive enzyme [Smith et al., 1996], whereas COX-2 is induced by LH surges in COCs [Richards, 2005]. COX-2-null mice exhibit a severe defect in COC expansion and decreased rates of both ovulation and fertilization [Davis et al., 1999].

Progesterone (P4) is a progestin hormone that is crucial for female reproduction in mammals; a close association between P4 and the establishment and maintenance of pregnancy is well known. P4 acts via its receptor, the P4 receptor (PR, official name Pgr), which is a nuclear receptor transcription factor that activates target genes by dimerizing and subsequently binding to gene promoter regions [Klein-Hitpass et al., 1990].

A role for PR in the uterine and mammary glands was demonstrated using PR-null mice. A remarkable morphological difference between the PR-null and wild-type mice was observed in these tissues [Lydon et al., 1995]. The importance of PR in the ovary was also reported, as PR expression in the granulosa cells was induced by LH surges in the rat [Park and Mayo, 1991] and mouse [Rose et al., 1999]. Notably, no follicle rupture occurred in PR-null mice [Robker et al., 2000]. Although the involvement of PR in the ovarian expression of numerous genes at the time of ovulation is well recognized, little is known about the mechanisms by which PR regulates the expression of

ovulation-related genes.

To elucidate the regulatory mechanisms of ovulation, I used the medaka (*Oryzias latipes*), a small fresh-water teleost known to be a useful animal model for various studies, particularly those investigating ovulation. Mature female medaka maintained under 14-h light/10-h dark cycles at 26-27°C has a 24-h spawning cycle in which oocyte maturation and ovulation occur. In the preovulatory follicle, a thin follicle layer encloses the oocyte. The innermost follicle layer consists of a single layer of the GCs that directly interact with the egg membrane (EM) of the oocyte. In addition, there is also a single layer of TCs associated with a single layer of the outermost ovarian epithelial cells and a basement membrane between the GC and TC layers.

The timing of the reproductive processes such as germinal vesicle breakdown (GVBD) and follicle rupture have been determined in previous studies [Iwamatsu, 1978]. Furthermore, *in vitro* ovulation systems with preovulatory follicles can also be used to investigate these processes [Ogiwara et al., 2010]. In addition, the medaka genome sequence [Kasahara et al., 2007] has been determined and is publicly available. Thus, the medaka system has several advantages, and intensive studies addressing ovulation mechanisms have been carried out in our laboratory using this model system. The results have revealed that at least three matrix metalloproteinases (MMPs), gelatinase A (official name mmp2), MT1-MMP (official name mmp14), and MT2-MMP (official name mmp15), are involved in follicle rupture during ovulation in medaka [Ogiwara et al., 2005]. These MMPs have been identified as essential for degradation of the extracellular matrix (ECM) present in the follicle layer. It was also observed that induction of *MT2-MMP* expression takes place in the medaka preovulatory follicles during ovulation [Ogiwara et al., 2013].

Recent studies from our laboratory have revealed that another proteolytic system is also required for successful ovulation in fish [Ogiwara et al., 2012]. The urokinase-type plasminogen activator (uPA, official name Plau-1)/plasmin system is activated in the preovulatory follicle prior to the activation of the MMPs described above, and the resulting activated plasmin participates in follicle layer degradation. Laminin, a major component of the basement membrane in the follicle layer, was determined to be a target of plasmin. The uPA/plasmin pathway is regulated by the endogenous protease inhibitor, plasminogen activator inhibitor-1 (Pai-1), which is synthesized and secreted from the granulosa cells of preovulatory follicles. *Pai-1* mRNA expression was shown to be under the direct control of the nuclear PR (nPR) induced by LH surges in the preovulatory follicles [Ogiwara et al., 2015].

As described above, the involvement of PGs in ovulation has been reported in a large number of studies conducted in mammalian species. Although studies addressing the role of PGs in teleost female reproduction are limited compared with studies in other mammals, the data implicate PGs, in particular, PGE_2 and $PGF_{2\alpha}$, in teleost reproduction. For example, $PGF_{2\alpha}$ is essential for successful *in vitro* ovulation in brook trout [Goetz and Garczynski, 1997]. It is also known that $PGF_{2\alpha}$ released into water by ovulating female goldfish stimulates male spawning behavior [Sorensen et al., 1988].

In our laboratory, experiments were carried out using medaka to determine whether PGs play a role in ovulatory processes [Fujimori et al., 2011; Fujimori et al., 2012]. As reported in these studies [Fujimori et al., 2011], a COX-2 inhibitor, indomethacin, suppressed *in vitro* follicle ovulation and the inhibitory effect of indomethacin on ovulation was completely nullified by PGE_2 addition. This result confirms that PGE_2 is involved in medaka ovulation. These studies also showed that *COX-2* mRNA and PGE_2

levels were relatively constant throughout the 24-h spawning cycle in medaka preovulatory follicles.

Six subtypes of PGE_2 receptors (EP1a, EP1b, EP2, EP3, EP4a and EP4b) were found in the medaka genome. Among these, *EP4b* (official name Ptger4b) was found to be dominantly expressed in the medaka preovulatory follicles [Fujimori et al., 2011]. A dramatic induction in *EP4b* mRNA expression in the follicle cells of preovulatory follicles has also been demonstrated [Fujimori et al., 2011; Fujimori et al., 2012].

Progestins have also been reported to play important roles during teleost ovulation. In teleosts, two types of progestins, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) and 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), have been identified [Nagahama and Yamashita, 2008]. It is well established that in early vitellogenic follicles, 17β-estradiol is actively produced in the ovaries. 17β-estradiol participates in the synthesis and secretion of vitellogenic protein in the liver and its uptake into the oocyte during growth [Senthilkumaran et al., 2004]. A dramatic shift from 17β-estradiol to DHP in steroidogenesis was demonstrated in salmonid fish [Senthilkumaran et al., 2004]. In medaka follicle cells, DHP levels are highest prior to oocyte maturation [Fukada et al., 1994], which is a well-established event that occurs during a dramatic elevation of DHP or 20β-S in the preovulatory follicle.

Among the two progestin hormone receptors, nPR and membrane PR (mPR), mPR is more heavily involved in reproduction [Nagahama and Yamashita., 2008]. mPR is reported to be a seven membrane-spanning receptor coupled to a Gi protein and is known to decrease intracellular cAMP levels in the oocyte when the receptor is activated by progestins. mPR knockdown experiments using morpholino antisense olignucleotides revealed that oocyte maturation is triggered by progestin hormones via

mPR activation in teleosts and amphibians [Nagahama and Yamashita, 2008]. On the other hand, only a few studies of nPR have been carried out in teleost species. Recently, nPR from the teleost zebrafish was characterized *in vitro* and was suggested to act as a transcription factor after binding to progestin [Hanna et al, 2010]. There have been no studies identifying any downstream gene(s) regulated by nPR in teleosts.

Based on the findings that PGE_2 was constitutively synthesized in the preovulatory follicles and that *EP4b* expression was dramatically induced in the follicles close to the initiation of ovulation, I assumed that the effect of PGE_2 on ovulation in medaka would be regulated through the expression of the receptor EP4b rather than through the synthesis of its ligand PGE_2. For this reason, the regulatory mechanism of EP4b induction in follicle cells was considered a worthwhile question for investigation. Because my preliminary experiment indicated that trilostane (TLS), an inhibitor of 3β-hydroxysteroid dehydrogenase, strongly inhibited the expression of *EP4b* mRNA, which was reproducibly induced by LH treatment of the control follicles, I hypothesized that progestins might play an important role in EP4b expression. Therefore, I decided to examine the possibility of progesterone hormone receptor involvement in EP4b induction. In this thesis, the importance of nPR and mPRs in the expression of a PGE_2 receptor subtype EP4b in the preovulatory follicles is described.

In Chapter 1, I described the induction of nPR by LH and subsequent *EP4b* expression via an LH-mediated pathway. Furthermore, I showed the co-expression of nPR and EP4b in the GCs of medaka preovulatory follicles. The inhibitory effect of RU486, an antagonist of nPR, was also described. Finally, direct binding of nPR to the promoter region of the EP4b gene approximately 12 h before ovulation was also demonstrated.

In Chapter 2, the possible involvement of mPR in *EP4b* expression was explored. The inhibitory effect of an activator of adenylate cyclase (AC), forskolin (FSK), on ovulation and on the LH-induced *EP4b* expression was described. Furthermore, expression of *mPRα* (official name, *paqr7*) and *mPRγ* (official name, *paqr5*) in the medaka ovary was presented. A reduction in intracellular cAMP levels via mPRα in response to DHP was also shown.

From these results, I conclude that nPR and mPRα are both involved in LH-induced EP4b expression in the preovulatory follicle GCs during ovulation in medaka.

Chapter 1

LH-induced expression of EP4b, a prostaglandin E_2 receptor indispensable for ovulation of the medaka *Oryzias latipes*, is regulated by a genomic mechanism involving nuclear progestin receptor

(The results presented in this chapter have been published in "Biology of Reproduction")

ABSTRACT

We previously reported that the prostaglandin E_2 receptor subtype EP4b plays a role in ovulation in a teleost species, medaka, and that *EP4b* mRNA is drastically induced in preovulatory follicles prior to ovulation. The present study focuses on the hormonal regulation of *EP4b* mRNA expression using this non-mammalian vertebrate model. Preovulatory follicles that had not been exposed to LH *in vivo* were incubated *in vitro* with medaka recombinant LH (rLH), which induced the *EP4b* mRNA expression. The addition of Trilostane (TLS), an inhibitor of 3β-hydroxysteroid dehydrogenase, strongly inhibited rLH-induced *EP4b* expression, and TLS-suppressed *EP4b* expression was restored to the level observed in rLH-treated follicles when 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) was included in the culture. I determined that the expression of the progestin-activated transcription factor nuclear progestin receptor (nPR) was also induced by medaka rLH in the follicle and that its expression preceded *EP4b* expression. Forskolin treatment induced both *nPR* and *EP4b* mRNA expression in the follicle. Follicular *EP4b* mRNA expression was drastically suppressed by RU486, which was demonstrated to compete with DHP for medaka nPR *in vitro*, suggesting a role for nPR in the expression of *EP4b* mRNA. A ChIP assay with preovulatory follicles isolated from spawning medaka ovaries demonstrated direct binding of nPR to the *EP4b* promoter. These results indicate that *EP4b* expression is regulated by a genomic mechanism involving nPR.

INTRODUCTION

In the process of ovulation, one or more viable oocytes are shed from mature ovarian follicles in response to a surge of LH [Espey and Richards, 2006; Lubzens et al., 2010]. This process is accomplished by the coordinated activities of many proteins and enzymes, many of which are induced to very high levels by the gonadotropin. Current evidence indicates that as many as 90 genes/proteins may be related to ovulation [Espey and Richards, 2006]. These proteins include steroidogenic enzymes [Chaffin et al., 1999; Chaffin et al., 2000], nuclear progestin receptor [Natraj et al., 1993; Robker et al., 2009], vascular endothelial growth factor [Koos., 1995; Hazzard et al., 1999], and MMPs and their inhibitors [Curry and Osteen, 2001; Curry et al., 2001, Hagglund et al., 1999; Brown et al., 2010].

PGs are a group of animal hormones that are biosynthesized from C20 unsaturated fatty acids by sequential enzymatic reactions involving COX and specific synthases [Narumiya et al., 1999; Smith and langenbach, 2001]. PGs exert a variety of biological activities by binding to their corresponding receptors [Simmons et al., 2004; Sugimoto and Narumiya, 2007]. The role of PGs in ovulation has been studied intensively in mammalian species [Espey and Richards, 2006; Orczyk and Berhman., 1972; Armstrong, 1981; Murdoch et al., 1993]. Gene targeting studies have demonstrated that at the time of ovulation, the dominant ovarian prostaglandin PGE_2 is produced by COX-2 and that the binding of this PG to its receptor EP2 results in the expansion of the COC [Hizaki et al., 1999; Richards et al., 2002]. The involvement of PGs in ovulation has also been documented for teleost fish [Cetta and Goetz., 1982; Patiño et al., 2003; Lister et al., 2008; Jalabert and Szölösi, 1975; Stacey and Pandey, 1975; Goetz and Nagahama, 1985; Kagawa et al., 2003], amphibians [Schuetz, 1986;

Chang et al., 1995; Ramos et al., 2008; Sena and Liu, 2008], and hens [Shimada et al., 1986]. However, the follicular tissue structures are considerably different between mammalian and non-mammalian species. COC is never formed in the ovaries of non-mammalian animals, suggesting that the role of PGs in non-mammalian vertebrate ovulation must be different from their role in COC expansion in mammalian ovulation. Little is currently known about the role of PGs in the process of non-mammalian ovulation.

The small freshwater teleost Medaka (*Oryzias latipes*) is an excellent model system for studies of reproductive biology, particularly of ovulation [Ogiwara et al., 2005; Ogiwara et al., 2010; Ogiwara et al., 2012; Ogiwara et al., 2013; Takahashi et al., 2013]. Our recent studies using this fish demonstrated that PGE_2 plays an indispensable role in ovulation as indicated by the strong suppression of the ovulation rate by the COX inhibitor indomethacin [Fujimori et al., 2011]. The data also demonstrated that COX-2 and the PGE_2 subtype 4 receptor EP4b most likely function as a PGE_2-producing enzyme and a PGE_2 receptor, respectively, responsible for the effect of PGE_2 on ovulation in fish [Fujimori et al., 2011; Fujimori et al., 2012]. Surprisingly, in contrast with previous observations on the inducible nature of the COX-2 gene at the time of ovulation in many species including mammals [Espey and Richards, 2006; Zou et al., 1999; Roberts et al., 2000; Grosser et al., 2002; Lister et al., 2009], *COX-2* mRNA expression is not up-regulated in the medaka preovulatory follicle upon ovulation. In contrast, the transcript levels of *EP4b* increase dramatically with impending ovulation [Takahashi et al., 2013]. Thus, EP4b might be a key regulator of medaka ovulation, but not COX-2. However, the *in vivo* mechanism of the induction of *EP4b* mRNA expression in preovulatory follicles remains unknown.

The aim of the present study was to gain insight into the endocrine regulation of *EP4b* expression in the preovulatory follicles that are destined to ovulate. Therefore, I used an *in vitro* culture system for the ovulation of postvitellogenic follicles in the presence of medaka rLH [Ogiwara et al., 2013]. The results demonstrated that medaka rLH is effective in inducing follicular *EP4b* expression. The current data demonstrate that the classic nPR is involved in the expression of *EP4b* mRNA.

MATERIALS AND METHODS

Animals and tissues

This study used adult orange-red variant medaka *Oryzias latipes*. The fish were maintained and acclimated to the artificial reproductive conditions (14 h light and 10 h dark, 26°C) as previously described [Fujimori et al., 2011]. As these fish have a 24 h spawning cycle, they ovulated each day at the start of the light period. In this study, ovulation hour 0 corresponds to the start of the light period. Ovaries, ovarian follicles, and other tissues were isolated from the spawning female fish or adult male fish. Animal culture and experimentation were conducted in accordance with the guidelines for animal experiments of Hokkaido University and were approved by the Committee of Experimental Plants and Animals, Hokkaido University.

In vitro culture of dissected follicles

In the fish having a 24 h spawning cycle, large preovulatory follicles (approximately 1.0 mm in size, postvitellogenic phase, stage IX-X) are exposed to LH *in vivo* around at 17 h before ovulation [Ogiwara et al., 2013], which triggers a series of events leading to

oocyte maturation and ovulation. In our *in vitro* culture experiments, the follicles that had not been exposed to LH *in vivo* were used so that *in vivo* LH effect would be excluded. The ovaries were removed from the fish 22 h before ovulation and the preovulatory follicles were immediately isolated by using forceps under a dissecting microscope. The follicles from three to five fish ovaries were pooled and divided into several groups. Approximately 20 follicles were used per incubation. The follicles were placed in a 35-mm diameter culture dish in 4 ml of 90% M199 medium containing 50 μM gentamycin (pH 7.4). Medaka rLH, TLS (Sigma-Aldrich, St. Louis, MO), DHP (Sigma-Aldrich), FSK (Sigma-Aldrich), or RU486 (also known as mifepristone) (Sigma-Aldrich) were added to the culture (Fig. 1). Incubation temperatures were 26-27°C. The duration of incubation varied depending on the purpose of the experiment. After incubation, the follicles and/or follicle layers were analyzed for the mRNA and/or protein expression levels of various genes at various time points. These genes of interest included COX-2, the EP4b receptor, the classic *nPR*, nPR, and cytoplasmic actin (*actb*, Actb). The rates of GVBD and ovulation were also determined.

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

Total RNA isolation and RT were conducted as previously described [Fujimori et al., 2011]. PCR amplification was performed using a *TaKaRa Ex Taq*® Hot Start Version kit (Takara, Tokyo, Japan) as previously described [Fujimori et al., 2011]. The complimentary DNA fractions prepared in this way were then used for cDNA cloning.

Medaka nPR cDNA cloning

A cDNA fragment containing the medaka *nPR* coding region was amplified by PCR

with the ovarian cDNA using KOD-Plus-Neo DNA polymerase (Toyobo, Tokyo, Japan). The PCR primers are listed in Table 1. The PCR conditions were 94ºC for 2 min, followed by denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, and extension at 68ºC for 3 min, for a total of 30 cycles. A 2243-bp fragment of *nPR* was obtained. The amplified products of *nPR* were gel-purified and ligated into pBluescript II KS (-) (Stratagene, La Jolla, CA), and sequencing confirmed that the nucleotide sequences of the inserted fragments contained the entire coding region.

Luciferase assay

A cDNA containing the entire coding sequence of medaka *nPR* was amplified by PCR with KOD-Plus-Neo DNA polymerase using the cDNA fragment inserted into pBluescript II KS(-) as a template. The primers used for this PCR are listed in Table 1. The PCR products were gel-purified and ligated into the *Eco*RV site of the pCMV vector tag4 (Stratagene), which had been digested with the same enzyme. The resulting vector, named pCMV-nPR, was confirmed by sequencing. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1× penicillin-streptomycin-amphotericin B suspension (Wako, Osaka, Japan). The cells were transfected with pCMV-nPR, pGL4-MMTV (Promega Corporation, Madison, Germany), a firefly luciferase expression vector containing a partial sequence of mouse mammary tumor virus (MMTV), and pRL, a *Renilla* luciferase expression vector. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, the medium was exchanged for fresh medium containing various concentrations of progestins. The progestins used in this

experiment were DHP, P4 (Sigma-Aldrich), and 20-S (Sigma-Aldrich). After the cells were cultured for 24 h, the luminescence of the sample was measured in a Lumat LB9507 (Berthold, Bad Wild Bad, Germany) using the Dual-luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to co-expressed *Renilla* luciferase activity.

The OLHNI-2 cell line derived from the caudal fins of adult medaka of the HNI inbred strain [Hirayama et al., 2006] was purchased from the Riken Bioresource Center Cell Bank (Tukuba, Japan) and cultured in L-15 medium (Invitrogen) supplemented with 10% FBS, 1× penicillin-streptomycin-amphotericin B suspension, 2 mM L-glutamine solution (Wako) and 10 mM Hepes buffer (Invitrogen) at 33°C. A 419-bp nucleotide corresponding to a region including the partial *EP4b* gene promoter (-419 to –1, AB859022 in the DDBJ/EMBL/GenBank databases) was inserted into the pGL3 firefly luciferase expression vector (Promega) (pGL3-EP4b). The cells were simultaneously transfected with the pGL3-EP4b vector described above and the other two expression vectors, pCMV-nPR and pRL, using ScreenFect^{TM} A (Wako). The cells were then incubated in the presence or absence of DHP.

Chromatin immunoprecipitation (ChIP)

Preovulatory follicles (approximately 200/one group) were isolated from spawning fish ovaries 12 h before ovulation, gently crushed in M199 medium with forceps and then centrifuged. The precipitates were suspended in 1 ml of phosphate buffered saline (PBS) and treated with trypsin (0.25% solution containing 1 mM EDTA) for 30 min at room temperature with gentle agitation. The reaction mixture was then centrifuged to obtain the precipitates. The precipitates were washed twice with L-15 medium (Invitrogen) and

passed through a 100 μm nylon filter (BD Biosciences, Bedford, MA). The resulting filtrates containing both GCs and TCs were fixed in L-15 medium containing 1% formaldehyde (Wako) for 10 min at room temperature. The fixation reaction was terminated by adding glycine to 0.125 M for 5 min, followed by sedimentation and washing with ice cold PBS several times. The samples were lysed with SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) containing 1×Protease Inhibitor Cocktail Set I (Wako) and 1×Phosphatase Inhibitor Cocktail Solution II (Wako), and incubated for 10 min on ice. The lysed materials were sonicated on ice 6 times for 10 seconds at 20% output with a 30 second interval using a SONIFIER 250 advanced (Branson Ultrasonics Corporation**,** Danbury, CT) to shear the chromatin DNA. The samples were then centrifuged at 13,000 ×g for 10 min, and the resulting supernatants were used for immunoprecipitation. A 5% portion of the supernatant was saved as an input control. The supernatants were diluted 1:9 in dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 0.01% SDS) and mixed with Protein G-Sepharose (GE Healthcare, Buckinghamshire, England) that had been previously treated with anti-medaka nPR antibodies for 2 h at 4°C. Non-immune IgG served as a negative control. After overnight incubation at 4°C, precipitates were obtained by centrifugation. The precipitates were washed one time each with low-salt buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS) and then high-salt buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS). After washing twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), the resulting precipitates were treated with 1% SDS/0.1 M $NaHCO_3$ at room temperature. After a 30 min incubation, the supernatants were obtained and treated with 0.2 M NaCl for 4 h at 65°C. Contaminating proteins

were digested with proteinase K (50 µg/ml) for 1 h at 50°C. The sheared DNA was finally recovered by DNA extraction. The DNA was analyzed with real-time PCR. To design PCR primers, I initially searched for the sequence corresponding to the 5'-flanking region of the *EP4b* gene using publicly available medaka genome databases (ENSORLG00000003829), and found that the sequence in the database was incomplete. In this way, the nucleotide sequence of the medaka *EP4b* gene 5'-flanking region was determined. An 8700 bp 5'-flanking region starting at the transcription start site (+1) has been submitted to the DDBJ/EMBL/GenBank databases under accession number AB859022. Five sets of primers were used (Table 1). The sheared DNA without antibody precipitation was processed by reverse cross-linking and served as an input control. As a positive control, the sheared medaka DNA precipitated with anti-human RNA polymerase II antibody (GTX80341, GeneTex Inc., Irvine, CA) was analyzed by real-time PCR using a set of primers (Table 1) for the medaka *gapdh* promoter.

Real-time RT-PCR

Complimentary DNA prepared as described above was used for real-time RT-PCR with a KAPA SYBR FAST ABI Prism qPCR kit (Kapa Biosystems, Woburn, MA) using an ABI 7300 Real-Time PCR System (Life Technologies, Inc., Rockville, MD). The PCR conditions and data analysis were determined according to previously reported procedures [Fujimori et al., 2011]. The gene transcript levels in the ovaries or ovarian follicles were normalized using *actb*. The primers used in this study are listed in Table 1.

Northern blot analysis

Northern blotting was performed by a previously reported method [Ogiwara et al., 2013]. Tissues of adult medaka fish were obtained 3, 12 or 21 h before ovulation. The total RNA was extracted from the tissues, and aliquots (60 μg) of the RNA were separated by formaldehyde agarose gel electrophoresis and blotted onto a Hybond-N^+ membrane (GE Healthcare). A cDNA fragment for *nPR* (nucleotides 1020-1574, registered no. AB854418) was used as a probe. The membrane was hybridized with the ^{32}P-labeled 555-bp probe. As a control, *actb* mRNA was detected with a ^{32}P-labeled 312-bp *actb* cDNA fragment [Fujimori et al., 2011].

In situ hybridization

Probes were obtained by RT-PCR with medaka ovary total RNA using primers for medaka *nPR* (Table 1). The amplified fragment was used to generate both antisense and sense digoxigenin (DIG)-labeled riboprobes with a DIG RNA Labeling Mix (Roche Diagnostics, Manheim, Germany). The ovaries isolated from mature medaka at 12 h before ovulation were frozen and sectioned at a thickness of 14 μm. The sections were fixed in paraformaldehyde at room temperature for 15 min and acetylated. Prehybridization, hybridization, washing of the sections, and detection with anti-DIG-alkaline phosphatase (AP) conjugate were performed as previously described [Fujimori et al., 2011].

medaka rLH

Medaka rLH was produced in Chinese hamster ovary (CHO) k-1 cells as previously described [Ogiwara et al., 2013] with a slight modification. In brief, cells stably expressing medaka rLH were cultured in F-12 medium (Wako) for 7 days. The

conditioned medium was then collected and concentrated 20-fold. The protein concentration of the concentrated rLH samples was 4 mg/ml. In this study, the rLH samples were used without further purification.

Preparation of antibodies

A 2004-bp cDNA coding for the complete nPR protein (667 residues) was generated by RT-PCR and ligated in-frame to the *Eco*RI and *Xho*I sites of the vector pET30a (Novagen, Madison, WI). The protein antigen was produced using the bacterial expression system in the pET30a vector. Recombinant proteins eluted from a Ni^{2+}-Sepharose column were injected into mice according to a previously described method [Ogiwara et al., 2013]. After four boosted injections of the antigen every 2 weeks, antisera were obtained. The antiserum and non-immune serum were used directly in western blot and immunohistochemical analysis. Specific nPR antibody was affinity-purified using a membrane on which pure antigen was blotted and used for the ChIP assay. Anti-medaka Actb was prepared as previously described [Ogiwara et al., 2012].

Western blot analysis

Intact large preovulatory follicles isolated from adult fish ovaries were immediately used for analysis. The follicles incubated *in vitro* with various reagents in the presence of medaka rLH were also analyzed. Intact or treated follicles were crushed with forceps in 50 mM Tris-HCl buffer (pH 8.0), and the resulting follicle tissues were centrifuged at 13,000 rpm for a few seconds. The precipitated tissues were suspended in the same buffer and centrifuged at 1,000 rpm for 5 min. After repeating this washing step, the

de-yolked tissues were lysed in 50 mM Tris-HCl buffer (pH 8.0) containing 1% SDS and used for SDS-PAGE/western blot analysis. Immunoblotting with Immobilon Poly Vinylidene DiFluoride (PVDF) membrane was performed following standard procedures. Diluted antisera for medaka nPR and Actb (control) were used as primary antibodies. Signals were detected using an Immobilon Western kit (Millipore, Bedford, MA). The protein concentration was determined using a Pierce BCA Protein Assay Reagent kit (Thermo Fischer Scientific, San Jose, CA).

Immunohistochemical analysis

Ovaries were dissected from spawning medaka 12 h before ovulation and fixed in 0.2 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde (Wako). After dehydration, the specimens were embedded in paraffin and sectioned at 5 μm thickness. Paraffin sections preparation, treatment with H_2O_2, and blocking with Block Ace (Dainippon Sumitomo Seiyaku Inc., Osaka, Japan) were performed as previously described [Ogiwara et al., 2012]. The sections were incubated with the primary antisera (anti-nPR, ×100 dilution in PBS) for 60 min and washed three times in PBS. The sections were then incubated for 60 min with a Dako EnVision+ System-HRP Labelled Polymer Anti-mouse (×4 dilution in PBS) (Dako, Copenhagen, Denmark) and washed three times in PBS. Signals were detected using an AEC kit (Vector Laboratories, Burlingame, CA). Sera obtained from normal mice were used as a control.

Phylogenetic analysis

All NPR/nPR sequences were searched for similarity using PSI Blast with medaka nPR using the web servers of the National Center of Biotechnology Information (NCBI).

They were aligned with the medaka nPR sequence by CLUSTALW, and percentage identity was calculated. A phylogenetic tree was constructed from the aligned sequences as previously described [Oka et al., 2013]. The NCBI gene IDs of sequences used are as follows: AB854418, NP_001159807, AAG42362, AAA49013, NP_000917, BAA89539, BAB85993, and BAF91193.

Statistical analysis

Results were expressed as the mean ± S.E.M. Data were analyzed using a one-way ANOVA followed by Tukey's post hoc test or a Student's *t*-test when comparing only two conditions. $P<0.05$ was considered statistically significant.

RESULTS

Effect of rLH on the expression of COX-2 and EP4b mRNA in the preovulatory follicle of the medaka ovary

Preovulatory follicles isolated from fish ovaries 22 h before ovulation, falling before the endogenous LH surge, were incubated in the presence of rLH (Fig. 1A). Exposure to the gonadotropin resulted in a slight increase in the expression of *COX-2* mRNA after approximately 21 h of incubation, although this increase was not significant (Fig. 1B). In contrast, the levels of *EP4b* mRNA were remarkably elevated by rLH, with a maximum at 18 h (Fig. 1C). Next, I examined the effect of TLS, an inhibitor of 3β-hydroxysteroid dehydrogenase [Potts et al., 1978], on rLH-stimulated *EP4b* mRNA expression. The addition of the inhibitor into the culture completely suppressed rLH-induced *EP4b* expression (Fig. 1D). TLS-suppressed *EP4b* expression was restored to the level observed for rLH-treated follicles when DHP was added to the culture

together with TLS.

Cloning of medaka nPR cDNA

Knowing that DHP is an essential factor for *EP4b* mRNA expression in the rLH-treated preovulatory follicle, I hypothesized that nPR might be involved in the rLH induction of *EP4b* expression. An Ensembl database [Ensembl Database, Genome Information. http://www.ensembl.org/index.html] search suggests that medaka contains one *nPR* gene. The medaka nPR sequence available from the NCBI Sequence Database [NCBI. Sequence Infromation. http://www.ncbi.nlm.nih.gov/] was found to be approximately 30 residues shorter than those of other vertebrates. Sequence alignment analysis revealed that the C-terminal half of the DNA-binding domain of nPR was deleted, suggesting that this putative protein might not be functional. Therefore, a *nPR* cDNA clone (2243 bp) was isolated from the ovary of spawning medaka in this study. This clone coded for a protein with a 667-residue amino acid sequence. Like NPRs/nPRs from other vertebrate species [Aranda and Pascual, 2001; Todo et al., 2000; Ikeuchi et al., 2002; Hanna et al., 2010; Chen et al., 2010; Liu et al., 2005; Katsu et al., 2008; Conneely et al., 1986; Misrahi et al., 1987], medaka nPR has five functional modules, an N-terminal A/B domain, the DNA-binding domain (C), a hinge region (D), and a ligand-binding domain (E). When the amino acid sequence of the fish nPR was compared with those of other species, the DNA-binding domain and ligand-binding domain exhibited high sequence homology: 91-97% amino acid identity for the DNA-binding domain and 66-85% amino acid identity for the ligand-binding domain (Fig. 2A). A phylogenetic analysis of NPRs/nPRs revealed that medaka nPR clustered together with those of eel and zebrafish (Fig. 2B), consistent with the results of previous studies [Hanna et al.,

2010; Chen et al., 2010].

Expression of nPR mRNA and nPR protein in medaka tissues

Transcripts of *nPR* (2.8 kb) were only detectable in the ovary at 12 h but not 3 h before ovulation (Fig. 3A), suggesting cyclic ovarian expression of this gene throughout the 24 h spawning period. A very weak signal was also observed in the testis. Expression of *nPR* mRNA in the ovary was greatest in the intermediate period of the 24 h spawning cycle. Strong *nPR* mRNA signals were detected in the ovaries of spawning medaka between 19 and 7 h before ovulation (Fig. 3B). Medium-sized follicles from -47 (vitellogenic phase, stage VII) to -31 h (postvitellogenic phase, stage VIII) of ovulation expressed little or no detectable *nPR* transcripts (Fig. 3C). However, large-sized follicles (postvitellogenic phase, stage IX-X) isolated within 24 h before ovulation displayed an increased expression of *nPR* mRNA starting at -19 h with a maximum at -11 h of ovulation. Western blot analysis using diluted anti-medaka nPR antiserum, but not normal serum, detected a 71.3 kDa protein in the extracts of preovulatory follicles and of the follicular layers 12 h before ovulation (Fig. 3D). nPR was detected in the layers between 11 and 3 h before ovulation (Fig. 3E). These results indicate that *nPR* mRNA and the protein are expressed in the large preovulatory follicles that are destined to ovulate.

Localization of nPR mRNA and nPR protein in the fish ovary

In situ detection of *nPR* mRNA was conducted using ovaries isolated 12 h before ovulation. The antisense probe for *nPR* exclusively stained the follicle layer of large preovulatory follicles (Fig. 4A, arrows in the left and middle panels). No detectable

staining was observed with the sense probe (Fig. 4A, right panel), indicating that the antisense probe was suitable for specific *nPR* mRNA detection.

I next detected nPR protein by immunohistochemical analysis using anti-medaka nPR antibody. Signals were found in the follicle layer of large-sized preovulatory follicles and in the oocyte cytoplasm of small growing follicles with diameters less than 300 μm (Fig. 4B, left panel). Enlarged images of the follicle layer revealed stronger positive signals in the GCs than in the TCs (Fig. 4B, middle panel), indicating that the GCs are responsible for the dominant portion of nPR expression. The control mouse serum exhibited no signal (Fig. 4B, right panel), confirming the specificity of the antibody.

In vitro induction of nPR mRNA and nPR protein expression by rLH in the preovulatory follicle

The levels of *nPR* mRNA expression were very low in the follicle incubated in the absence of rLH, whereas the transcript levels were dramatically enhanced by rLH, peaking 12 h after the start of the incubation (Fig. 5A). Synthesis of nPR in the follicles treated with rLH *in vitro* was confirmed by western blot analysis (Fig. 5B). These results indicate that similar to *EP4b*, the expression of *nPR* mRNA is induced by LH.

Effects of DHP and FSK on EP4b and nPR mRNA expression in the preovulatory follicle

I examined the effects of DHP, medaka MIH, and FSK, an activator of AC, on the expression of *EP4b* and *nPR* mRNA using an *in vitro* follicle culture system. For *EP4b* expression, the preovulatory follicles isolated at -22 h were incubated for 18 h with

DHP or FSK (Fig. 1A). DHP alone had no effect on the induction of *EP4b* expression in the follicle (Fig. 5C), whereas *EP4b* mRNA expression was induced by FSK. Follicles incubated with rLH exhibited reduced *EP4b* mRNA expression when DHP was included in the medium. To further examine the effect of the steroid hormone on the follicular *EP4b* expression, the time-courses of the receptor mRNA levels were compared using rLH-stimulated preovulatory follicles incubated with or without external addition of DHP (Fig. 5D). Expression of *EP4b* mRNA was induced in the rLH-stimulated follicles irrespective of whether DHP was externally added to the incubation medium. The maximal levels of *EP4b* expression were comparable between the follicles treated with rLH alone and those treated with both rLH and DHP. However, the follicular expression of *EP4b* mRNA occurred 6 h earlier in the presence of DHP; after a 6 or 12 h incubation, rLH-treated follicles expressed *EP4b* mRNA at higher levels in the presence of DHP compared with the follicles without DHP, while after 18 h reversed. These results indicate that external addition of DHP stimulates *EP4b* transcription in the rLH-treated follicles incubated for 6 and 12 h. DHP and FSK were also tested *in vitro* for their effects on the expression of *nPR* mRNA in the preovulatory follicle after 12 h of incubation. Treatment of the follicle with FSK but not DHP resulted in a drastic induction of *nPR* mRNA expression (Fig. 5E). The follicles incubated in the medium containing both rLH and DHP expressed *EP4b* mRNA at levels comparable to that in the presence of rLH. Unlike *EP4b* follicular expression, the time of *nPR* mRNA expression did not change to a detectable level (data not shown). Preovulatory follicles incubated with rLH for 30 h ovulated with an ovulation rate of approximately 75% (Fig. 5F), confirming our previous results [Ogiwara et al., 2013].

Neither DHP nor FSK induced follicle ovulation. The addition of DHP did not affect the *in vitro* ovulation rate of rLH-treated follicles.

The above results indicate that the activation of the cAMP pathway is closely associated with the induction of *EP4b* and *nPR* mRNA expression in the LH-stimulated follicles. These results also indicate that DHP may be an important factor contributing to the expression of *EP4b* mRNA in the follicle, but not *nPR* mRNA.

Role of nPR in the expression of EP4b mRNA in the preovulatory follicle

To further gain insight into the relationship between nPR and *EP4b* mRNA expression in the preovulatory follicle, I investigated whether the nPR agonist/antagonist RU486, which also acts as a powerful antiglucocorticoid and a weak antiandrogen in mammals [Spitz and Bardin, 1993; Song et al, 2004; Hodgson et al, 2005], may affect *EP4b* expression in the medaka follicle. To this end, I first characterized medaka nPR for its specificity toward the three progestins DHP, P4, and 20-S by a reporter gene assay using HEK293T cells transfected with pCMV-nPR pGL4-MMTV and pRL vectors. All progestins induced luciferase activity via nPR (Fig. 6A) with EC_{50} values of 2.97±0.58 (10^{-8} M, n=7) for DHP, 2.04±0.24 (10^{-7} M, n=7) for P4, and 3.52±0.72 (10^{-7} M, n=7) for 20 β-S. Next, I examined whether RU486 might interact with nPR. The addition of RU486 to the medium at concentrations greater than 10^{-6} M resulted in the inhibition of DHP-activated luciferase activity (Fig. 6B). The value of IC_{50} was calculated to be 4.45±0.64 (10^{-6} M, n=7). These results suggest that this compound acts as a nPR antagonist.

rLH-stimulated *in vitro* follicle ovulation was suppressed as the RU486 concentration was increased; ovulation was inhibited approximately 90% by 10^{-4} M

RU486 (Fig. 6C). In the presence of 10^{-4} M RU486, rLH-induced follicular expression of *EP4b* mRNA was strongly inhibited (Fig. 6D). No difference was observed in the expression level of nPR between the RU486-treated and control follicles after the 12 h incubation (data not shown), indicating that RU486 had no effect on the expression of nPR in the rLH-stimulated follicles. RU486 also had no effect on the extent of oocyte maturation (Fig. 6E).

Evidence for the binding of nPR to the promoter region of the EP4b gene

I examined the possibility that nPR directly binds to the promoter region of *EP4b* by performing a ChIP assay using the preovulatory follicles isolated 12 h before ovulation (Fig. 7A). Among the five primer sets used, nPR recruitment to the promoter was observed with the primer set 1 (designed to amplify a 120 bp fragment from -242 to -123) and primer set 2 (designed to amplify a 125 bp fragment from -893 to -769). The binding activity of nPR to the -200 bp region was approximately 3 times greater than that to the -800 bp region. Using the primer set 1, ChIP assays were further conducted with the preovulatory follicles isolated 16, 12, and 4 h before ovulation. The 120 bp nucleotide fragment was amplified in follicles obtained 12 h before ovulation (Fig. 7B). Finally, I examined the binding of nPR to the *EP4b* promoter using OLHNI-2 cells. The cells were simultaneously transfected with three expression vectors, pCMV-nPR, pGL3-EP4b and pRL and then treated with or without DHP. A dose-dependent increase in luciferase activity did not occur with increasing nPR concentration in the absence of DHP (Fig. 7C). The transcriptional activity was detected with the extracts of cells incubated with DHP, suggesting that the nPR-mediated transcription of the medaka *EP4b* gene is ligand-dependent. I conducted similar *in vitro* transcriptional assay

experiments using mammalian cells including COS-7 cells, HEK293T cells and CHO cells, but failed to detect luciferase activities in any of these cell extracts (data not shown).

DISCUSSION

This study investigates the mechanism of drastic induction of *EP4b* mRNA expression in the preovulatory follicle upon medaka ovulation. The results demonstrate that the expression of *EP4b* mRNA in the follicle is induced by the gonadotropin LH using the *in vitro* follicle culture system. Furthermore, these data show that rLH-induced expression of *EP4b* mRNA was mediated by the transcription factor nPR.

Previous studies using gene knockout technology in mice demonstrate that NPR is essential for LH-dependent follicle rupture during ovulation in mice [Lydon et al., 1995]. Subsequent studies revealed that the expression of many ovulation-related genes is under the control of NPR in mammals [Robker et al., 2009]. The above findings derived from studies using mammalian species prompted us to investigate the role of nPR in LH-induced expression of the *EP4b* gene. Several results in this study highlight the importance of nPR in follicular *EP4b* mRNA expression: i) *nPR*/nPR and *EP4b* were both induced in the follicle by rLH treatment *in vitro*; ii) the depletion of intrafollicular DHP, the presumed physiological nPR ligand in medaka [Sakai et al., 1987; Fukuda et al., 1994], by TLS treatment drastically decreased *EP4b* expression; iii) the expression of *nPR*/nPR preceded the *EP4b* mRNA expression in the preovulatory follicle both *in vitro* and *in vivo*; iv) the level of rLH-induced *EP4b* mRNA expression was drastically reduced when the preovulatory follicle was treated with the nPR antagonist RU486; and more importantly, v) direct binding of nPR to the promoter region of the *EP4b* gene was

observed.

Two NPR protein isoforms exist in mammals, NPR-A and NPR-B. These isoforms are generated from a single *NPR* gene due to the presence of two distinct translation initiation sites [Couse et al., 2006]. In medaka, only one form of nPR is translated. A single-band 2.8 kb transcript in northern blot analysis and a 71.3 kD polypeptide in western blot analysis were reproducibly detected. [Hanna et al., 2010] detected the nPR protein with a molecular mass of 69 kD in zebrafish ovaries.

A growing body of evidence indicates that DHP acts through binding to cell surface membrane-bound progestin receptors (mPRs) to initiate rapid intracellular signaling and biological responses [Thomas et al., 2012]. A recent study demonstrated that in addition to a critical physiological role in the meiotic maturation of fish oocytes, mPRs also act as intermediaries in progestin signaling in the follicle cells surrounding the oocyte in Atlantic croaker [Dressing et al., 2010]. Indeed, I observed that in the medaka ovary, transcripts of mPRα but not mPRβ (official name, *paqr8*) or mPRγ were expressed in the somatic cell layers of the preovulatory follicles (our unpublished observation). The mPRα mRNA expression levels were found to be relatively constant in the preovulatory follicle during the 24 h spawning cycle of the medaka, indicating that mPRα may always be present on the surface of the follicle cells. However, as shown in Fig. 5C, the treatment of the preovulatory follicles with DHP alone did not induce *EP4b* mRNA expression. These findings argue against the idea that mPRα may play a role in *EP4b* expression in the follicle cells, although I cannot presently completely exclude the possibility of mPRα involvement.

As mentioned above, *EP4b* mRNA expression follows that of *nPR* in the preovulatory follicles. The level of *nPR* mRNA was found to be the highest

approximately 11 h before ovulation in a 24 h spawning cycle, whereas *EP4b* mRNA levels peaked at around the time of ovulation *in vivo* [Fujimori et al., 2011]. In the *in vitro* culture of the preovulatory follicles in the medium supplemented with rLH, an approximately 6 h delay of *EP4b* mRNA expression was observed compared with the timing of *nPR* expression (Chapter 1). Our current data indicate that similarly to rLH, FSK induced the expression not only of the *nPR* gene but also of the *EP4b* gene. These findings suggest that rLH-induced expression of these genes requires the activation of AC, which synthesizes cAMP. This idea is consistent with the general belief that the LH receptor signals predominantly via Gs to activate AC and increase intracellular cAMP levels in a wide variety of vertebrate species including mammals [Hunzicker-Dunn and Mayo, 2006], chickens [Mizutani et al, 1998], and teleosts [Oba et al., 1999; Vischer and Bogerd, 2003; Chauvigné et al., 2012].

RU486 is a well-known nPR antagonist that inhibits ovulation in mice [Loutradis et al., 1991] and rats [Van der Schoot et al., 1987; Sanchez-Criado et al., 1990; Gaytan et al., 2003]. Inhibition of ovulation by the same compound was recently reported for *Xenopus laevis* [Dhillon et al., 2010]. In the present study,I observed that RU486 inhibited medaka ovulation in a dose-dependent manner using the *in vitro* rLH-induced ovulation system of medaka preovulatory follicles. Because the gonadotropin-induced follicular expression of *EP4b* mRNA was drastically suppressed by RU486 treatment and because EP4b has been recently demonstrated to play an essential role in fish ovulation [Fujimori et al., 2011; Fujimori et al., 2012], it is reasonable to speculate that the reduced ovulation rates of RU486-treated follicles would be caused, in part, by the deterioration in the LH-induced synthesis of EP4b involving the transcription factor nPR. In contrast, the rate of GVBD was not affected

by RU486 treatment, suggesting that mPRs involved in the meiotic maturation of fish oocytes may be insensitive to the antagonist. This finding is consistent with recent studies documenting that RU486 does not bind to sea trout or human mPRα [Thomas et al., 2007; Dressing et al., 2012]. Previous studies have established that RU486 is also a powerful anti-glucocorticoid and a weak anti-androgen in mammals [Spitz and Bardin, 1993; Song et al., 2004; Hodgson et al., 2005]. Inhibition of follicle ovulation and LH-induced *EP4b* mRNA synthesis by RU486 is probably not attributed to its anti-glucocorticoid or anti-androgen activities.

Note our finding that external addition of DHP advanced the time of *EP4b* mRNA expression in the rLH-treated follicle *in vitro*. This effect of the external addition of DHP can be explained as follows. Once nPR was generated in the follicle cells in response to rLH, the progestin receptor was able to immediately associate with DHP, which was present in large amounts in the culture. This would promptly trigger the transcription of the *EP4b* gene. In contrast, in the culture of rLH-treated follicles without externally added DHP, the follicle cells would take some time to produce DHP to a high enough level to activate nPR. I presume that differences in the availability of DHP would have led to differences in the timing of follicular *EP4b* mRNA expression under these two different conditions. The advancement of the timing of *EP4b* expression by extrinsic DHP in the rLH-treated follicle suggests a role of this steroid hormone in nPR-mediated expression of the *EP4b* gene.

DNA binding by steroid receptors is generally ligand-dependent [Abdel-Hafiz and Horwitz, 2014], although some human genes have been reported to be regulated by un-liganded NPR [Jacobsen et al., 2005]. Our current data are consistent with the idea that medaka nPR acts as a transcription factor for *EP4b* gene expression in a

ligand-dependent manner. Our finding that fish nPR requires DHP to exert its effect on the expression of *EP4b* in the *in vitro* luciferase assay using OLHNI-2 cells is relevant for this hypothesis. However, it remains to be determined whether liganded nPR also binds to the promoter region of the *EP4b* gene upon transcription under *in vivo* conditions. In this context, our attempts to examine whether medaka nPR acts on *EP4b* transcription in a DHP-dependent or DHP-independent manner were not successful if mammalian cells were used. This may be due to the lack of species-specific transcription factor(s) necessary for medaka *EP4b* expression.

In vivo binding of nPR to the *EP4b* promoter was demonstrated by a ChIP assay using the preovulatory follicles isolated 12 h before ovulation. The current data indicate that nPR may bind to the *EP4b* promoter at -893 to -123. There is no perfect palindromic progesterone response element (PRE) site [Hill et al., 2012; Jacobsen and Horwitz, 2012] in the promoter of the medaka *EP4b* gene. However, I found one potential PRE half-site (5'-TGTTCT-3' located at –329 to –323) in the sequence. This fact leads us to speculate that the binding of nPR to the PRE half-site might be an important step in the induction of *EP4b* mRNA expression in follicles predicted to ovulate. Further detailed studies are required to determine whether this site is indeed critical for nPR-induced expression of the *EP4b* gene in the follicles.

I found that FSK treatment of the preovulatory follicles induced *nPR*/nPR and *EP4b* expression. This observation implies that this compound appears to mimic the actions of LH. However, the follicles treated with FSK failed to ovulate, indicating that FSK is not potent enough to induce the follicles to ovulate. It is obvious that numerous genes must be activated in the preovulatory follicle to ensure successful ovulation. Activation of all ovulation-related genes could be accomplished by LH stimulation, but

only a limited number of such genes were activated when the preovulatory follicles were treated with FSK alone. Note that in rat [Clements et al., 1998] and mouse GCs [Sriraman et al., 2003], the expression of *NPR* mRNA is induced by FSK. This fact strongly suggests a common mechanism governing the expression of *NPR/nPR* gene expression throughout vertebrates. However, unlike mammalian *NPR*, the induction of *nPR* mRNA by FSK was not blocked by the A-kinase inhibitor H-89 in the follicles of the fish (our unpublished observations), indicating the presence of a distinct mechanism of gene expression for the progestin receptor in fish. I am currently conducting studies addressing the detailed endocrine mechanism of *nPR* expression in medaka.

Our previous [Fujimori et al., 2011; Fujimori et al., 2012] and current data allow us to propose a model for the expression of the EP4b receptor in the LH-primed medaka preovulatory follicle destined to undergo ovulation (Fig. 8). Postvitellogenic follicles in the fish ovary undergo LH stimulation at approximately 17 h before ovulation [Ogiwara et al., 2013]. The LH receptor, which is localized on the surface of GCs and TCs of the follicles [Ogiwara et al., 2013], is activated by the gonadotropin. This brings about Gsα activation, followed by AC activation, resulting in an increase in the intracellular cAMP concentration. A rise in cAMP concentration in the follicle cells simultaneously leads to the activation of at least two biological pathways: steroidogenesis and *nPR* gene expression. The steroidogenic pathway, which is active in the production of 17β-estradiol in the follicle before the LH surge, would be modulated to actively produce DHP [Sakai et al., 1987; Iwamatsu, 1980], with the maximal DHP level occurring approximately 14 h before ovulation [Sakai et al., 1988]. There is a general understanding that both the GCs and TCs are involved in the synthesis of the steroid hormone in teleost fish, including salmonids [Nagahama and Yamashita, 2008], but in

this model, I assume that GCs alone are capable of producing DHP at this stage. A previous study documented that medaka denuded oocytes underwent maturation in response to gonadotropin when co-cultured with isolated GCs [Nagahama and Yamashita, 2008]. In parallel, transcription and translation of the *nPR* gene are elicited by increased intracellular cAMP levels in the follicle cells. This event most likely occurs in the GCs, as nPR has been demonstrated to be associated with these cells (Fig. 4B). nPR produced within several hours after the LH surge may bind with DHP, which is presumed to be present at a high enough level in the follicle at this time [Sakai et al., 1988]. Activated nPR, in turn, functions as a critical transcription factor for *EP4b* gene expression. However, direct evidence for the binding of DHP-bound nPR to the promoter *in vivo* has not been provided in support of this model. No information is currently available on when EP4b appears on the cell surface of the GCs, as the protein could not be detected. However, our previous study using the antagonist as a receptor suggests that EP4b functions at or shortly before the time of ovulation [Fujimori et al., 2012]. In this model, based on our previous finding that the COX-2 protein was mainly localized to the TCs of the large preovulatory follicle by immunohistochemical analysis [Takahashi et al., 2013], I assume that TCs predominantly produce PGE_2.

A large number of ovulation-related genes induced by LH have been identified in mammals [Espey and Richards, 2006; Robker et al., 2009]. In teleosts, various genes and gene products are known to be induced at or around the time of ovulation: trout ovulatory protein-2 mRNA [Garczynski and Goetz, 1997; Schalburg et al., 2005], amago salmon 20β-hydroxysteroid dehydrogenase activity [Nagahama, 1997], medaka *star* and *cyp17a1* mRNA [Nagahama and Yamashita, 2008], rainbow trout serine proteinase 23, *adam22*, *cscl14*, *fgf2*, and *ace2* mRNA [Bobe et al., 2006], and channel

catfish *cyp11a*, *cyp17a1*, *cyp19*, and *hsd3b* mRNA [Zmora et al., 2007]. More recently, we have identified *EP4b* [Takahashi et al., 2013; Fujimori et al., 2011] and *mmp15* [Ogiwara et al., 2013] as ovulation-related genes in medaka. The present study provides evidence that nPR is involved in the expression of *EP4b* mRNA in medaka ovaries. To the best of our knowledge, *EP4b* is the only gene that has been experimentally verified to be under the control of nPR during ovulation in any teleost species. A recent study has demonstrated that RU486 acts as an antagonist of zebrafish nPR and that it inhibits DHP-stimulated 11-ketotestosterone production in the testis [Chen et al., 2010], suggesting that 11β-hydroxysteroid dehydrogenase was stimulated by DHP via nPR

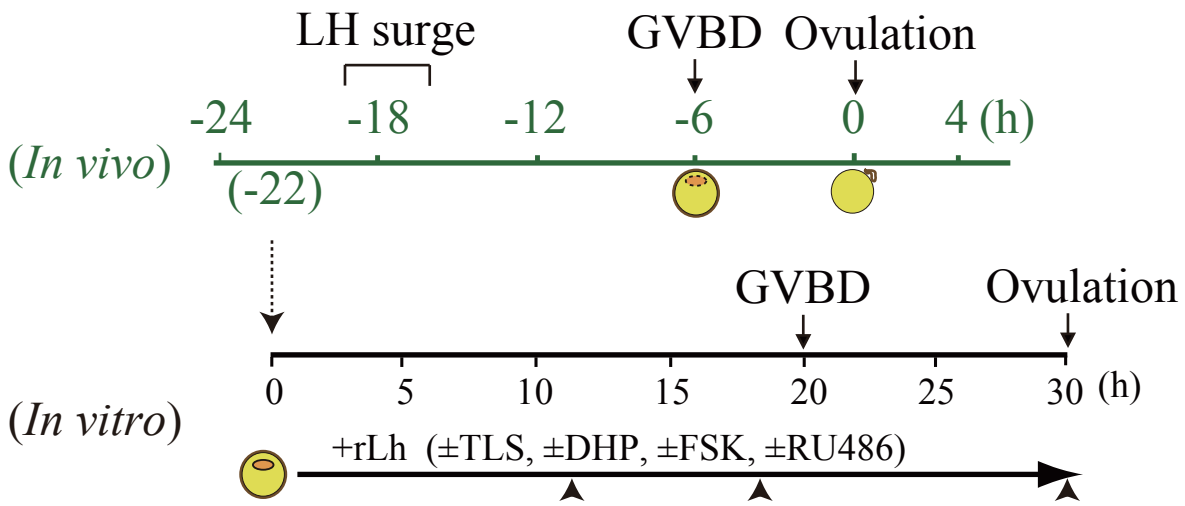
In summary, this study explored the mechanisms of *EP4b* mRNA expression by employing an *in vitro* follicle ovulation system in medaka. Our data indicate that *EP4b* mRNA expression is dramatically induced by rLH- and nPR-dependent mechanisms in the GCs of preovulatory follicles nearing ovulation. To the best of our knowledge, this study is the first to report that nPR is directly involved in the expression of the ovulation-related gene *EP4b* for teleost species. Information obtained from the present study will help to elucidate the molecular basis of *EP4b* gene expression in the GCs of preovulatory follicles in medaka.

Table 1. Primer nucleotide sequences used in chapter1

Gene	Primer sequences, Forward (F) and Reverse (R) primer (5'-3')	Accession No.
For Real-time RT-PCR		
COX-2	F:AAGCCCTATGCGTCTTTGA	AB516993
COX-2	R:CGGGGTTTCTCAACCAGTAG	
EP4b	F:CAGATGGTGATCCTGCTCAT	AB563504
EP4b	R:GCCAGGAGGTCTTCATTGAT	
nPR	F:AGAGGAACCCATCACCCTTC	AB854418
nPR	R:TGAGGACTCCATAGTGGCAA	
bact	F:TGACGGAGCGTGGCTACTC	D89627
bact	R:TCCTTGATGTCACGGACAATTT	
For cDNA cloning		
nPR	F:ACAAGAAGCTGCAACTTCTTCC	AB854418
nPR	R:GGAGACTTTATTGATCGATGC	
For luciferase assay		
nPR	F:ATGGAGAGTAAAATG	AB854418
nPR	R:TCACTTTGTGTGGAA	
EP4b	F:GGATAAGAGGAAGTCACT	AB859022
EP4b	R:ACTCAGACCAACCCAGCT	
For *in situ* hybridization/Northern blotting		
nPR	F:TCACATGCGGCAGCTGTAAAG	AB854418
nPR	R:AGCGCCAGCCCAGAGAGAACA	
For recombinant protein preparation		
nPR	F:CCGGAATTCATGGAGAGTAAAATGAAC	AB854418
nPR	R:CCGCTCGAGTCACTTTGTGTGGAACAG	
For ChIP assay		
EP4b (-0.2 kbp)	F:GACTTCCACCGTCATAGATC	AB859022
EP4b	R:TCCCTGGAGTCCAGAGTTCC	
EP4b (-0.8 kbp)	F:AGTCAATGTACCTATCAGAA	AB859022
EP4b	R:TTCCGTTTAACTTCAGTCAA	
EP4b (-1.4 kbp)	F:CGGTGAAATTGAGTTTGACA	AB859022
EP4b	R:AGTAACAGCTGTTTATTCTC	
EP4b (-2.0 kbp)	F:CCAGCTGCAATCAGAATGCT	AB859022
EP4b	R:GCAGAGACCCAATCTGTACA	
EP4b (-8 kbp)	F:GCCTCAAGGATTTTGAGTAA	AB859022
EP4b	R:TTTAACTCATCTCTACCTCT	
gapdh	F:TCATGCTTTAGCAATTGGAG	ENSORLG00000006033
gapdh	R:GTATGGGATTCACACTCACA	

Figure 1

A


LH surge
GVBD
Ovulation
-24
-18
-12
-6
0
4 (h)
(In vivo)
(-22)
GVBD
Ovulation
0
5
10
15
20
25
30
(h)
(In vitro)
+rLh (±TLS, ±DHP, ±FSK, ±RU486)

B

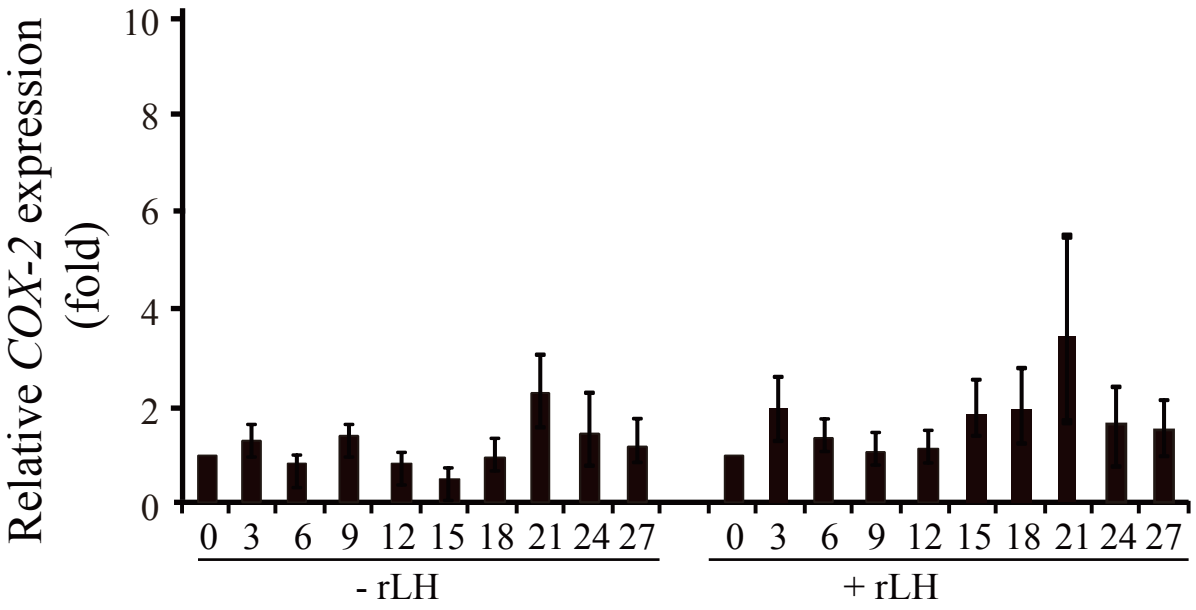
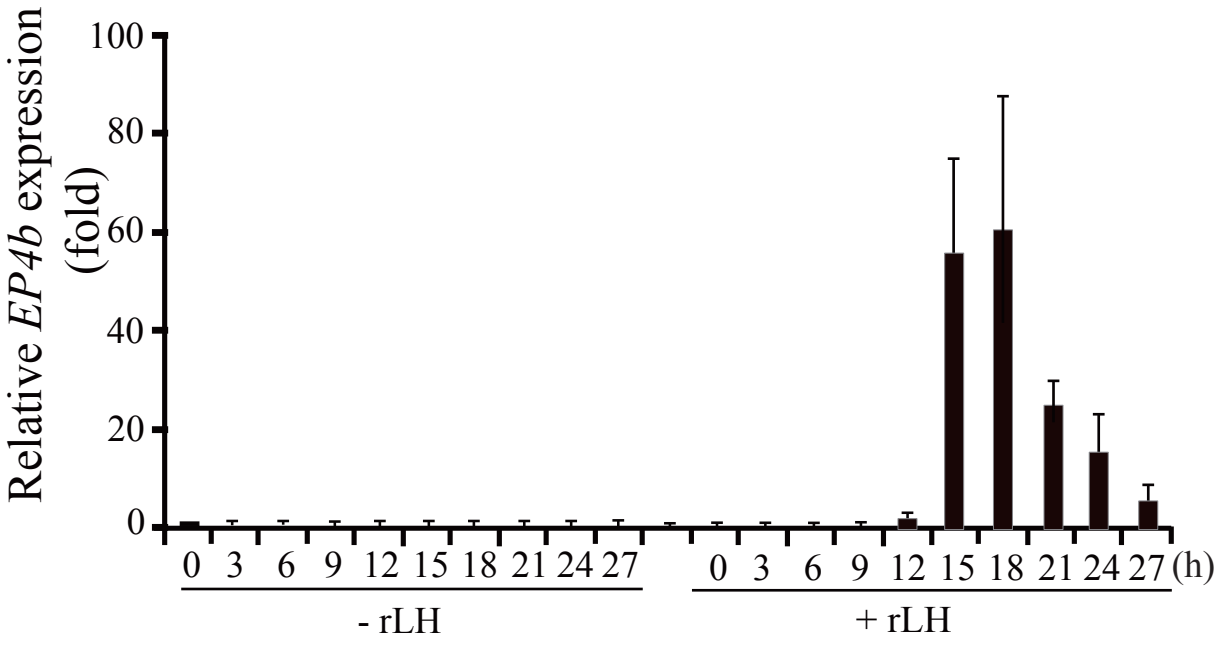

Relative COX-2 expression
(fold)
10
8
6
4
2
0
0 3 6 9 12 15 18 21 24 27
- rLH
0 3 6 9 12 15 18 21 24 27
+ rLH

Figure 1(Continued)

C



D

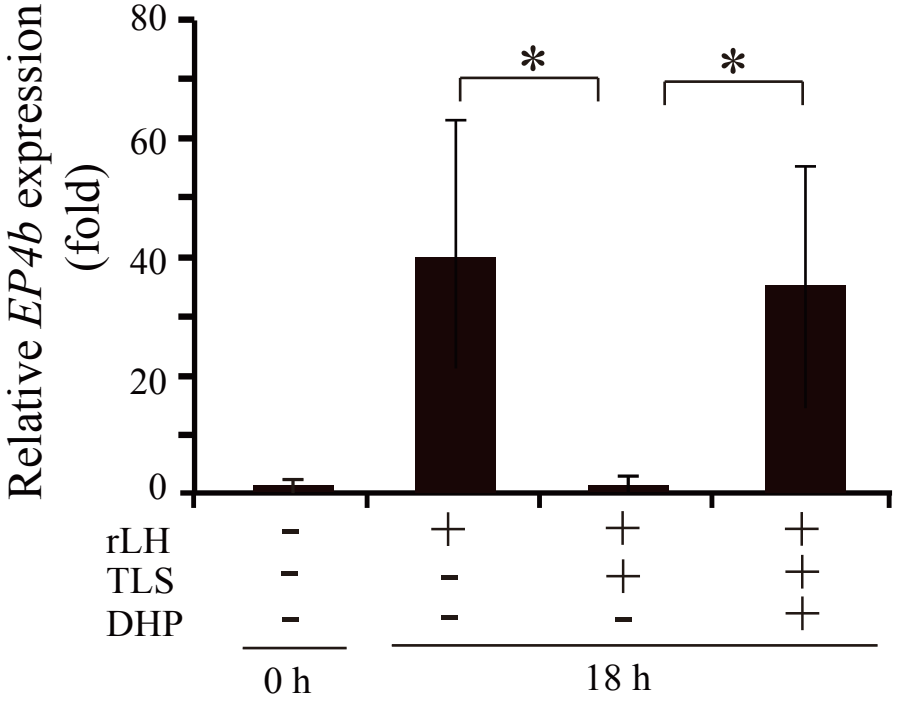


Figure 1. Induction of *EP4b* mRNA expression in the preovulatory follicles by rLH treatment.

(A) An outline of preovulatory follicle culture experiments carried out in this study. The follicles were isolated from spawning medaka ovaries 22 h before ovulation and incubated with mock-conditioned medium or medaka rLH (100 μg/ml). The expected timing of the LH surge, GVBD, and ovulation *in vivo* and *in vitro* are shown on the upper line. GVBD and ovulation time under *in vitro* culture conditions are also shown. TLS, trilostane; DHP, 17α, 20β-dihydroxy-4-pregnen-3-one; FSK, forskolin. Arrowheads indicate the time points of analysis. (B) Follicles cultured *in vitro* in the absence or presence of rLH (100 μg/ml) were collected every 3 h to extract total RNA. Real-time RT-PCR was conducted for transcripts of *COX-2*. The expression levels were normalized to those of *actb* and expressed as the fold change compared to the levels of the -22 h follicles. The results are presented as the mean ± S.E.M. of five separate experiments. (C) Follicles cultured *in vitro* in the absence or presence of rLH (100 μg/ml) were collected every 3 h to extract total RNA. Real-time RT-PCR was conducted for transcripts of *EP4b*. The expression levels were determined as in (B). The results are presented as the mean ± S.E.M. of five separate experiments. (D) Preovulatory follicles isolated from spawning medaka ovaries at 22 h before ovulation were incubated in the presence of rLH (100 μg/ml). In some experiments, TLS (25 μM) or TLS (25 μM) + DHP (0.1 μM) were also included in the culture. After 18 h of incubation, total RNA was extracted from the follicles for real-time RT-PCR analysis of *EP4b* mRNA. The fold change compared to the levels of the 0 h follicles are presented as the mean ± S.E.M. of five separate experiments. Asterisks denote significant differences ($P<0.05$).

Figure 2

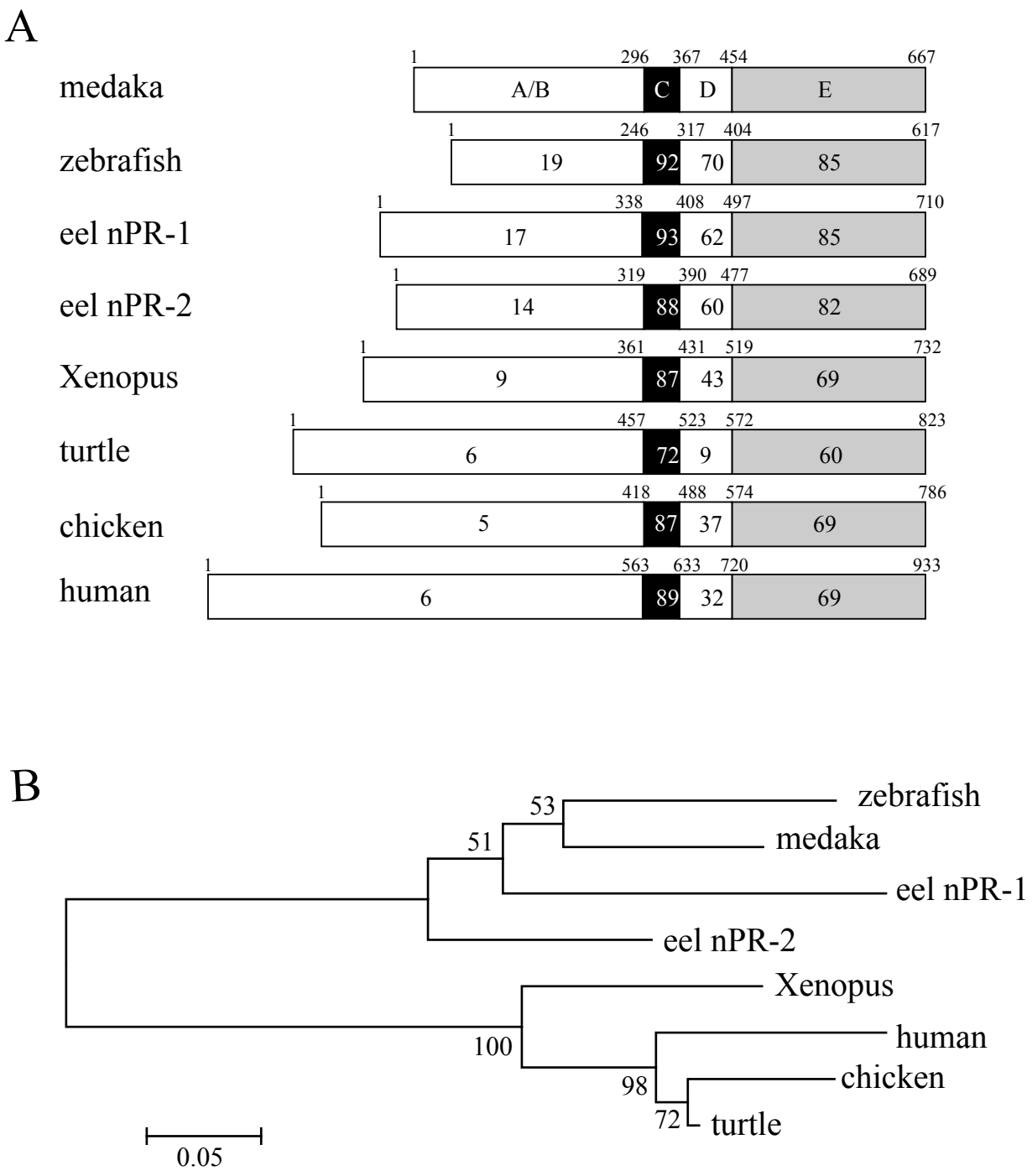

A
medaka
1
296
367
454
667
A/B
C
D
E
zebrafish
1
246
317
404
617
19
92
70
85
eel nPR-1
1
338
408
497
710
17
93
62
85
eel nPR-2
1
319
390
477
689
14
88
60
82
Xenopus
1
361
431
519
732
9
87
43
69
turtle
1
457
523
572
823
6
72
9
60
chicken
1
418
488
574
786
5
87
37
69
human
1
563
633
720
933
6
89
32
69
B
zebrafish
medaka
eel nPR-1
eel nPR-2
Xenopus
human
chicken
turtle
53
51
100
98
72
0.05

Figure 2. Comparison of medaka nPR with the counterpart proteins from some other vertebrate species.

(A) Amino acid sequence identities in the A/B, C, D, and E domains of medaka nPR and those of other species are indicated. The numbers within the domains represent percent identities compared with the sequences of the corresponding domains of medaka nPR. The numbers on each box indicate the residue numbers of the respective receptor proteins. (B) Phylogeny of vertebrate NPRs/nPRs. The number on each branch indicates the branch support value estimated in the PhyML program. The scale bar indicates that 0.05 amino acid substitutions are expected per site.

Figure 3

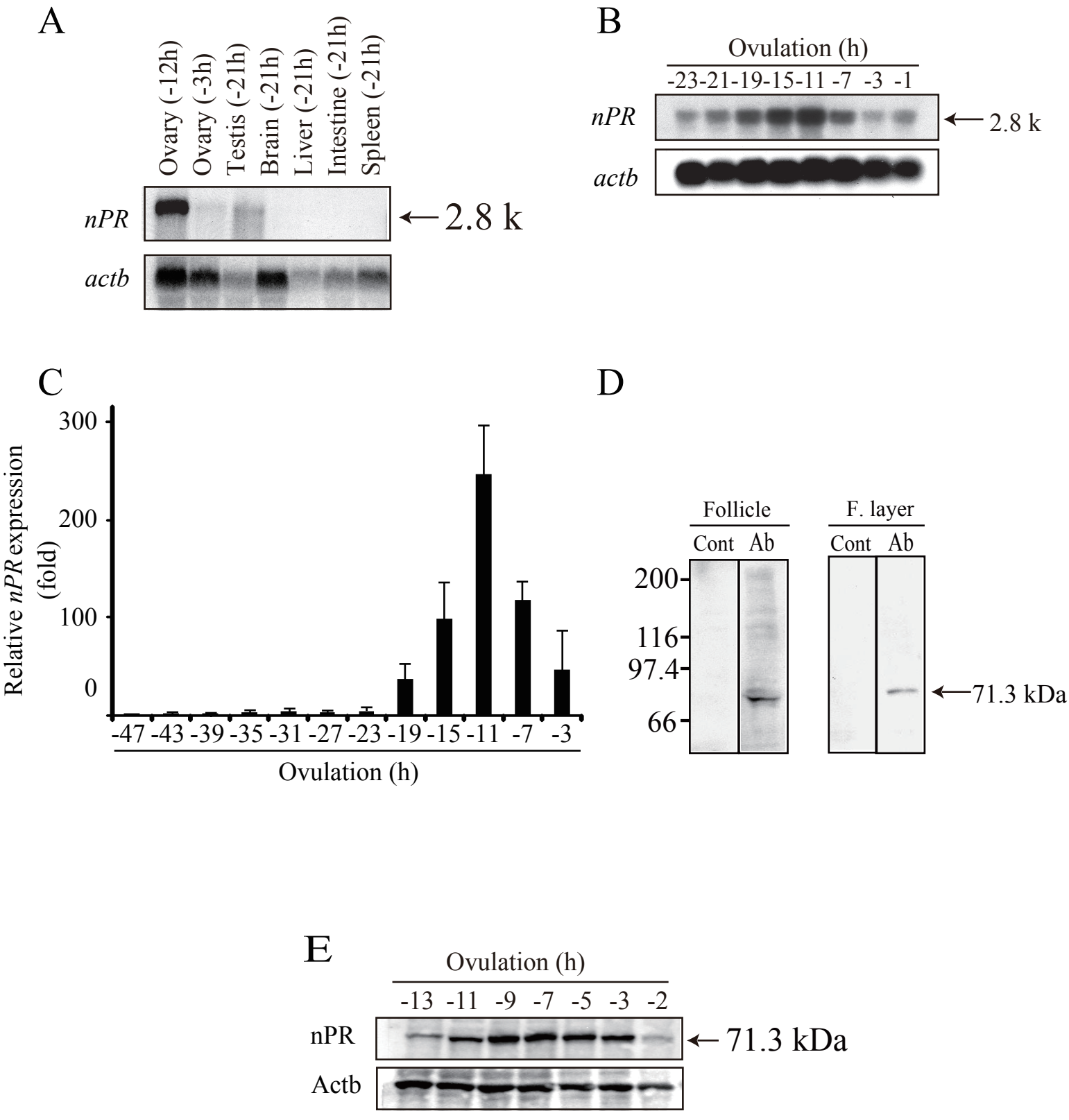

A
Ovary (-12h)
Ovary (-3h)
Testis (-21h)
Brain (-21h)
Liver (-21h)
Intestine (-21h)
Spleen (-21h)
nPR
actb
2.8 k
B
Ovulation (h)
-23 -21 -19 -15 -11 -7 -3 -1
nPR
actb
2.8 k
C
Relative nPR expression (fold)
300
200
100
0
-47 -43 -39 -35 -31 -27 -23 -19 -15 -11 -7 -3
Ovulation (h)
D
Follicle
F. layer
Cont Ab
Cont Ab
200
116
97.4
66
71.3 kDa
E
Ovulation (h)
-13 -11 -9 -7 -5 -3 -2
nPR
Actb
71.3 kDa

Figure 3. Expression of *nPR* mRNA and nPR protein in the medaka ovary.

(A) Northern blot analysis of *nPR* mRNA was conducted using the total RNA of various medaka tissues. The tissues used were isolated from adult fish at 3 h (ovary), 12 h (ovary) or 21 h (testis, brain, liver, intestine, and spleen) before ovulation in a 24 h spawning period. *actb* transcripts were measured as a control. Aliquots of 60 μg of total RNA were loaded per lane. The reproducibility of the findings was confirmed by conducting two separate experiments, and the results of one experiment are presented. (B) Northern blot analysis of *nPR* mRNA was conducted using the total RNA isolated from large preovulatory follicles of spawning female fish ovaries. *actb* transcripts were detected as a control. The reproducibility of these findings was confirmed by conducting two separate experiments, and the results of one experiment are presented. (C) Real-time RT-PCR for *nPR* was conducted using the total RNA (2.5 μg each) isolated from ovarian follicles every 4 h during the last 48 h before ovulation. Follicles isolated at 47, 43, 39, 35, 31, and 27 h before ovulation were in the late vitellogenic phase, whereas those at 23, 19, 15, 11, 7, and 3 h before ovulation were in the postvitellogenic phase. The expression levels of *nPR* mRNA were normalized to that of *actb* and expressed as the fold change versus the level of the -47 h follicle. The results are presented as the mean ± S.E.M. of five separate experiments. (D) Preovulatory follicles were isolated from spawning fish ovaries 12 h before ovulation and used to prepare tissue extracts of whole follicles (left panel) or follicle layers (right panel). SDS-PAGE/western blot analysis was performed using a specific anti-medaka nPR antibody. The antiserum (Ab) and non-immune serum (Control) were used after 1,000-fold dilution in PBS. Samples of approximately 25 μg and 5 μg per lane were loaded on the gel for the whole follicle and layer extracts, respectively. The

reproducibility of these results was confirmed by conducting three separate experiments, and the results of one experiment are presented. (E) Preovulatory follicles were isolated at the indicated time points from the spawning fish ovaries, and the follicle layers were then obtained by physical dissection. Extracts of the follicle layers were prepared and subjected to SDS-PAGE/western blotting using the nPR antibody. Aliquots of 5 μg of protein were applied per lane. Actb protein was also detected as a control. Three separate experiments were performed to confirm the reproducibility of the findings, and the results of one experiment are presented.

Figure 4

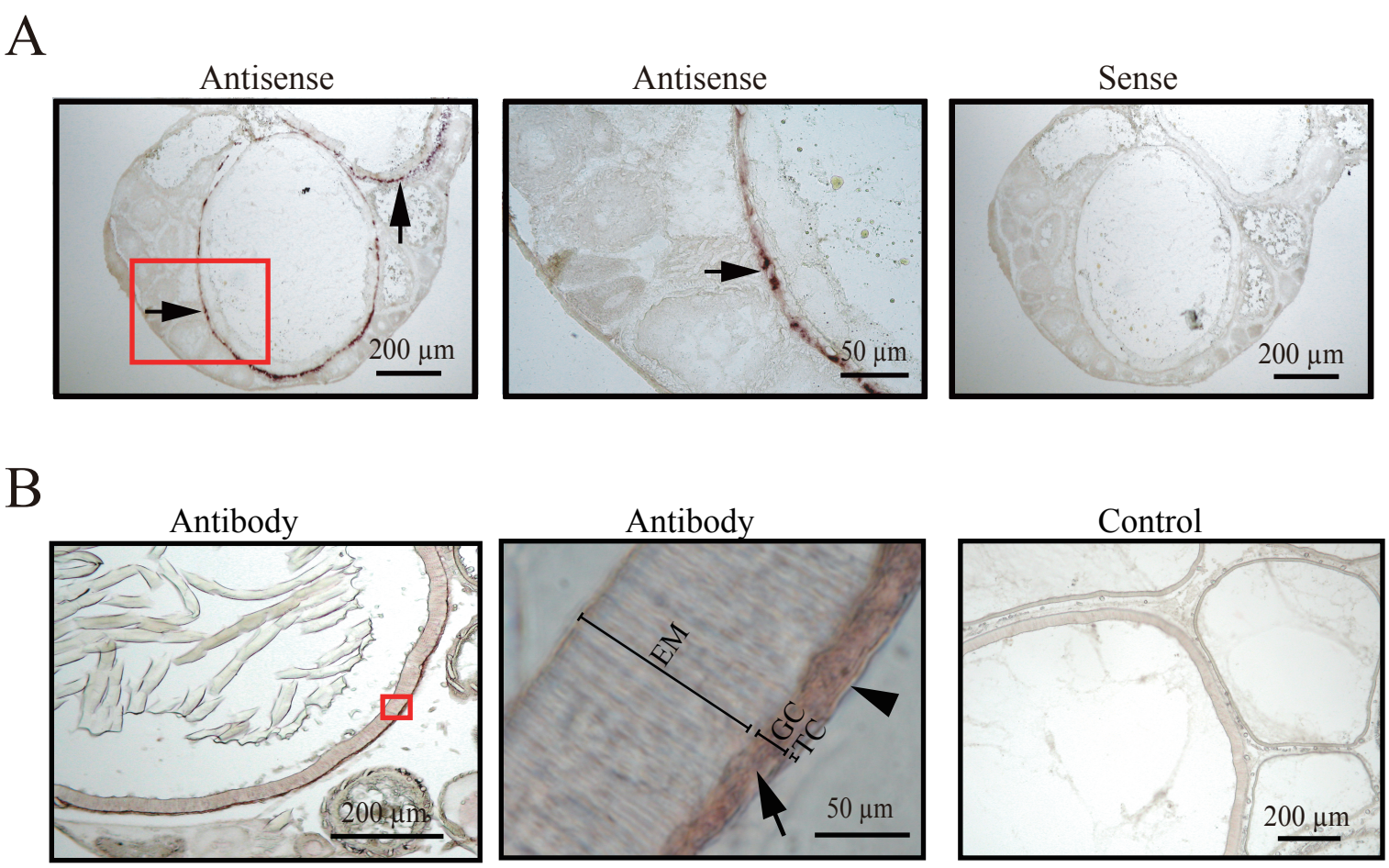

A
Antisense
Antisense
Sense
200 µm
50 µm
200 µm
B
Antibody
Antibody
Control
200 µm
EM
GC
TC
50 µm
200 µm

Figure 4. *In situ* localization of *nPR* mRNA and immunological localization of nPR protein in the medaka ovary.

(A) *In situ* hybridization analysis was conducted using frozen sections of ovaries isolated 12 h before ovulation. Antisense (left and middle panels) and sense probes (right panel) were used to detect signals. The boxed area in the left panel is shown at a higher magnification in the middle panel. Specific signals for *nPR* transcripts are indicated by arrows with the antisense probe. The reproducibility of the results was confirmed by repeating experiments three times, and the results of one experiment are presented. (B) Immunohistochemical analysis was conducted using paraffin sections of the ovaries isolated 12 h before ovulation. Anti-medaka nPR antiserum (left and middle panels) and non-immune serum (right panel) were used as primary antibodies at 100-fold dilutions in phosphate-buffered saline. The boxed area in the left panel is presented at a higher magnification in the middle panel. The middle panel shows the GC layer (arrow) and TC layer (arrowhead), both of which are stained positively. The reproducibility of the results was confirmed by repeating experiments three times, and the results of one experiment are presented. EM, egg membrane; GC, granulosa cell layer; TC, theca cell layer.

Figure 5

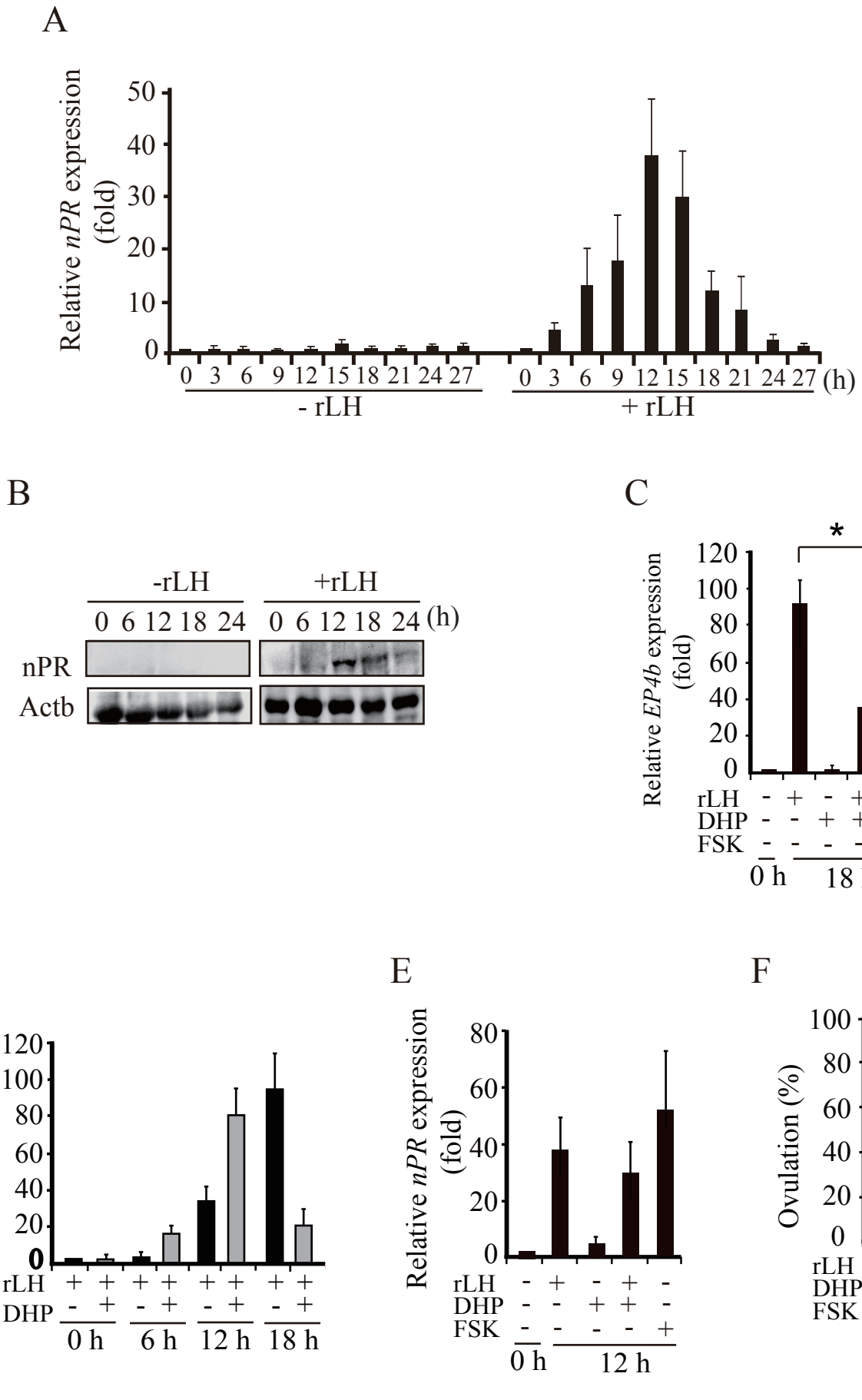

A
Relative nPR expression (fold)
50
40
30
20
10
0
0 3 6 9 12 15 18 21 24 27
0 3 6 9 12 15 18 21 24 27 (h)
- rLH
+ rLH
B
-rLH
+rLH
0 6 12 18 24
0 6 12 18 24 (h)
nPR
Actb
C
*
Relative EP4b expression (fold)
120
100
80
60
40
20
0
rLH - + - + -
DHP - - + + -
FSK - - - - +
0 h
18 h
D
Relative EP4b expression (fold)
120
100
80
60
40
20
0
rLH + + + + + + + +
DHP - + - + - + - +
0 h
6 h
12 h
18 h
E
Relative nPR expression (fold)
80
60
40
20
0
rLH - + - + -
DHP - - + + -
FSK - - - - +
0 h
12 h
F
Ovulation (%)
100
80
60
40
20
0
rLH - + - + -
DHP - - + + -
FSK - - - - +
0 h
30 h

Figure 5. Effects of rLH, DHP, and FSK on the expression of *EP4b* and *nPR* mRNA in the preovulatory follicle.

(A) Preovulatory follicles were isolated from spawning medaka ovaries 22 h before ovulation and incubated with medaka rLH (100 μg/ml). Total RNA was extracted from the *in vitro* incubated follicles taken every 3 h and used for real-time RT-PCR analysis of *nPR* mRNA. The expression levels were normalized to that of *actb* and expressed as the fold change compared to the levels of the -22 h follicles. The results are presented as the mean ± S.E.M. of five separate experiments. (B) The follicles cultured with or without rLH were collected at the indicated time points. After the yolk was removed by pressing and then washing the follicles several times, the residual follicle tissues were used for extraction. The extracts were analyzed by SDS-PAGE/western blotting using a specific antibody for medaka nPR. The reproducibility of the results was confirmed by repeating experiments three times, and the results of one experiment are presented here. (C) Preovulatory follicles isolated from spawning medaka ovaries 22 h before ovulation were incubated with or without rLH. DHP (0.1 μM) or FSK (1 μM) were added to the incubation medium. After 18 h of incubation, total RNA was extracted from the follicles and used for real-time RT-PCR of *EP4b* mRNA. The expression levels were normalized to *actb* mRNA expression. The fold changes compared to the levels of the 0 h follicles are presented as the mean ± S.E.M. of five separate experiments. Asterisks denote significant differences ($P<0.05$). (D) Preovulatory follicles isolated from spawning medaka ovaries 22 h before ovulation were incubated with rLH in the presence or absence of DHP (0.1 μM). After 0, 6, 12 or 18 h incubation, real-time RT-PCR of *EP4b* mRNA was conducted. The total RNA extraction, normalization of the expression levels and data presentation (n=5) are as in (C). (E) The preovulatory follicles were cultured as

in (C) except that the incubation time was 12 h. The total RNA was isolated from the follicles and used for real-time RT-PCR of *nPR* expression. After the expression levels were normalized to *actb*, the fold changes compared to the levels of the 0 h follicles were determined. These results are presented as the mean ± S.E.M. of five separate experiments. (F) The ovulation rate was determined for the follicles cultured in media containing various substances for 30 h. These results are presented as the mean ± S.E.M. of five separate experiments.

Figure 6

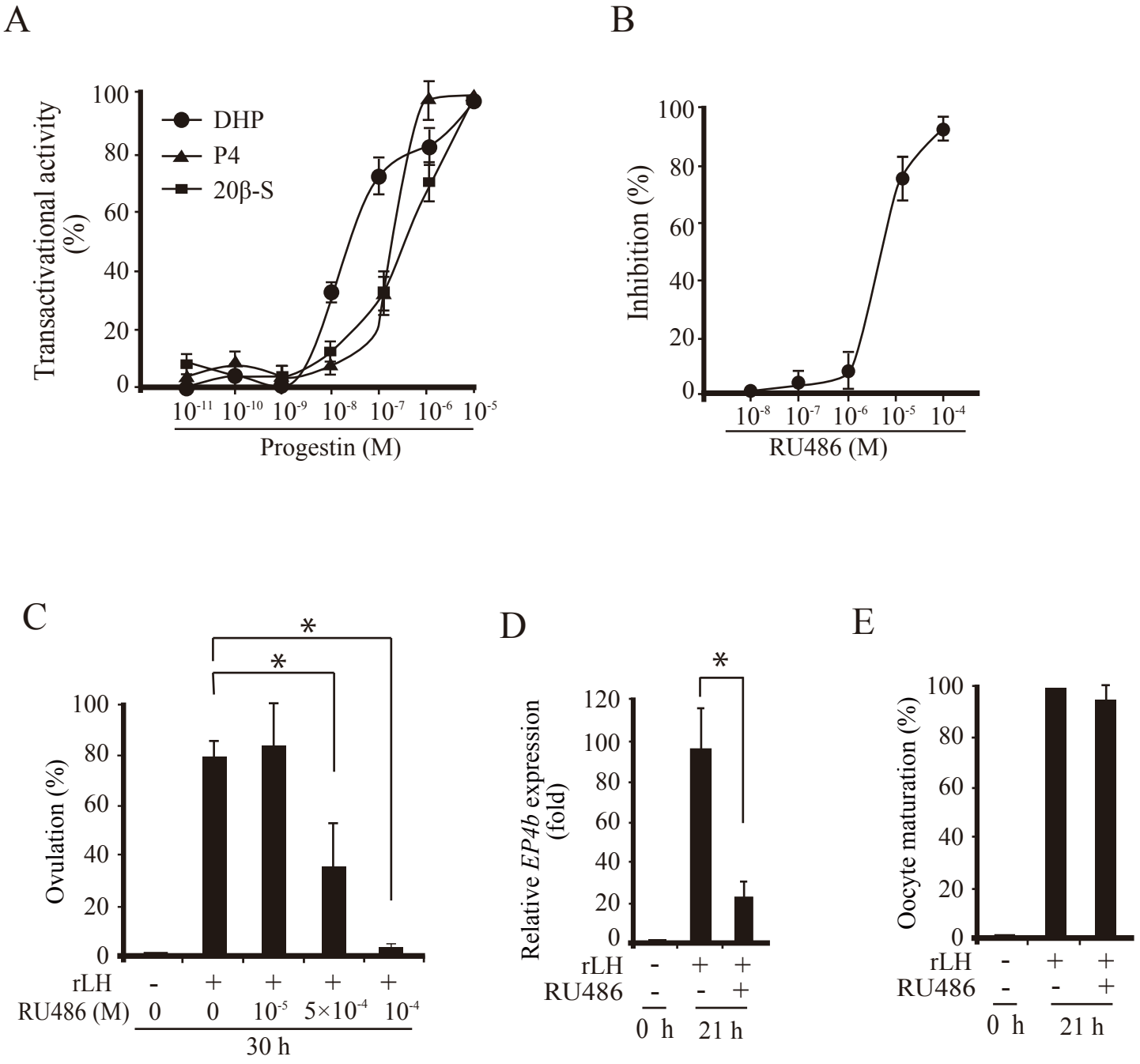

A
Transactivational activity (%)
DHP
P4
20β-S
Progestin (M)
B
Inhibition (%)
RU486 (M)
C
Ovulation (%)
rLH
RU486 (M)
30 h
D
Relative EP4b expression (fold)
rLH
RU486
0 h
21 h
E
Oocyte maturation (%)
rLH
RU486
0 h
21 h

Figure 6. Effect of RU486 on *EP4b* expression, oocyte maturation, and ovulation in the preovulatory follicle.

(A) HEK293T cells were transiently cotransfected with the pCMV-nPR and pGL4-MMTV vectors. The cells were incubated for 24 h with increasing concentrations of progestins. Extracts of the treated cells were then prepared and assayed for luciferase activity. Relative enzyme activities are shown by setting the activity of 10^{-5} M treated cells as 100. Each point represents the mean ± S.E.M. of seven independent experiments. (B) HEK293T cells transiently cotransfected with the pCMV-nPR and pGL4-MMTV vectors were incubated in media containing DHP (1 μM) and increasing concentrations of RU486 (10^{-8} to 10^{-4} M) for 24 h. Extracts of the treated cells were then prepared and assayed for luciferase activity. Relative enzyme activities were determined, and the results are expressed as the percent inhibition of the transactivational activity of nPR by RU486. Each point represents the mean ± S.E.M. of seven independent experiments. (C) Preovulatory follicles isolated from spawning medaka ovaries 22 h before ovulation were incubated in the presence of rLH with increasing concentrations of RU486 for 30 h, and then the rate of ovulation was then determined. The results are presented as the mean ± S.E.M. of six separate experiments. Asterisks denote significant differences (P<0.05). (D) Preovulatory follicles isolated from spawning medaka ovaries 22 h before ovulation were incubated with rLH alone or with rLH + RU486 (10^{-4} M) for 21 h, and real-time RT-PCR analysis of *EP4b* mRNA expression was conducted using the total RNA isolated from the follicles. After normalizing the expression levels to *actb*, the fold changes compared to the levels of the 0 h follicles were determined. These results are presented as the mean ± S.E.M. of five separate experiments. The asterisk denotes significant differences (P<0.05). (E) Preovulatory follicles were treated as in (D) for 21

h and assessed for oocyte maturation. The rates of completion of GVBD were determined, and the results are presented as the mean ± S.E.M. of five separate experiments.

Figure 7

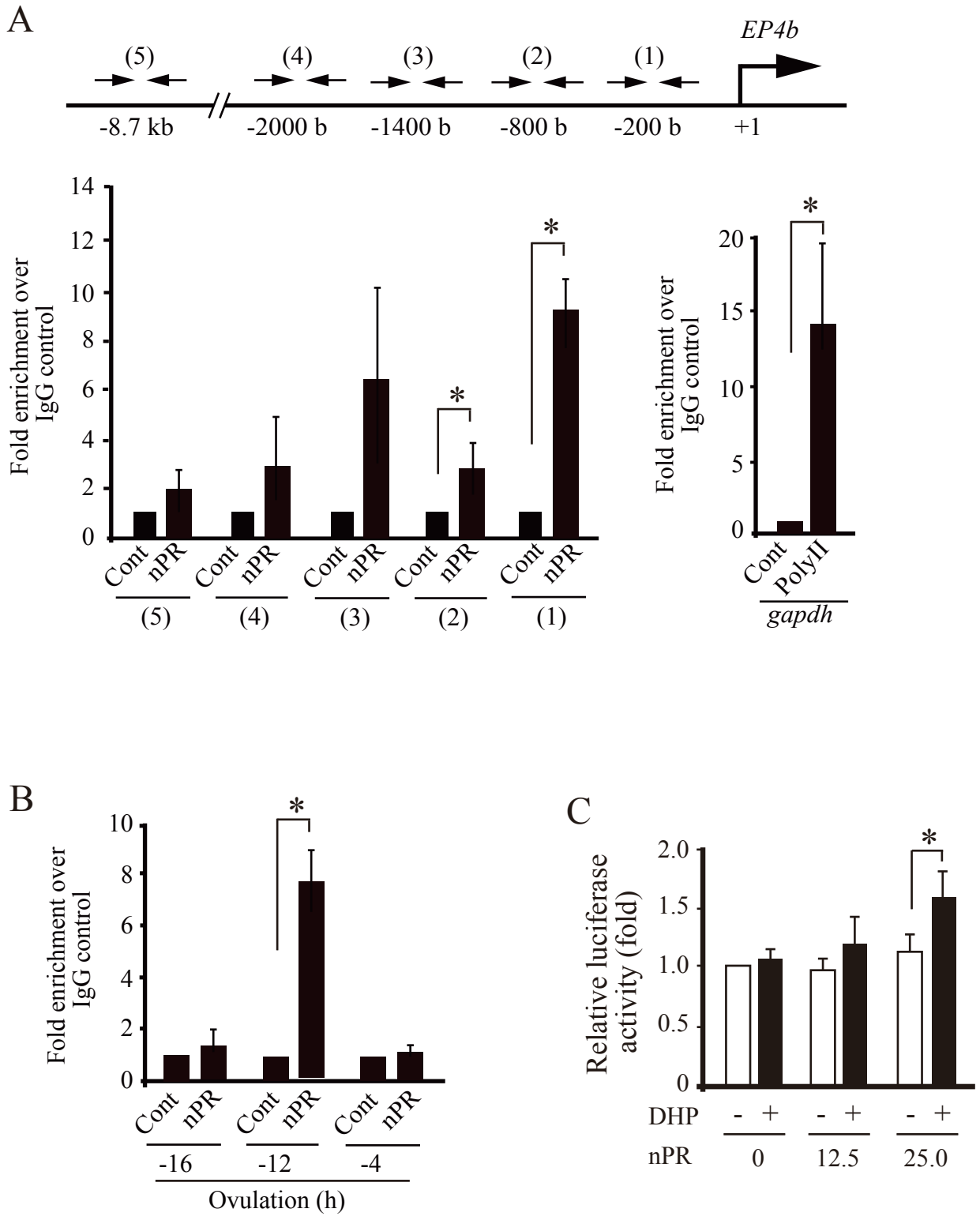

A
EP4b
(5)
(4)
(3)
(2)
(1)
-8.7 kb
-2000 b
-1400 b
-800 b
-200 b
+1
Fold enrichment over IgG control
Cont
nPR
PolyII
gapdh
B
-16
-12
-4
Ovulation (h)
C
Relative luciferase activity (fold)
DHP
nPR
0
12.5
25.0

Figure 7. Binding of nPR to the promoter region of the *EP4b* gene.

(A) Preovulatory follicles isolated 12 h before ovulation were used for a ChIP assay. The sheared DNA immunoprecipitated with anti-medaka nPR antibody was analyzed by PCR. The diagram in the top panel illustrates the positions of five ChIP primer sets in the 8.7-kb region upstream of the transcription start site of the *EP4b* gene. The transcription start site of the gene is indicated as +1. The suitability of the method was confirmed by the binding of RNA polymerase II to the promoter of the *gapdh* gene. The results are presented as the mean ± S.E.M. of four separate experiments. Asterisks denote significant differences ($P<0.05$). (B) Preovulatory follicles isolated 16, 12 or 4 h before ovulation were used for a ChIP assay using the primer set 1. Other procedures were the same as in (A). The results are presented as the mean ± S.E.M. of three separate experiments. The asterisk denotes significant differences ($P<0.05$). (C) Transcription assays were conducted using medaka OLHNI-2 cells. The cells were transiently co-transfected with pGL3-EP4b and pRL, along with increasing amounts of pCMV-nPR vector. After 24 h incubation, the cells were further treated with or without DHP (1 μM) for 24 h. Extracts of the treated cells were assayed for luciferase activity. Relative enzyme activities are shown by setting the activity of the extract without nPR (0 ng) and DHP at 1. The results are presented as the mean ± S.E.M. of six separate experiments. The asterisk denotes significant differences ($P<0.05$).

Figure 8

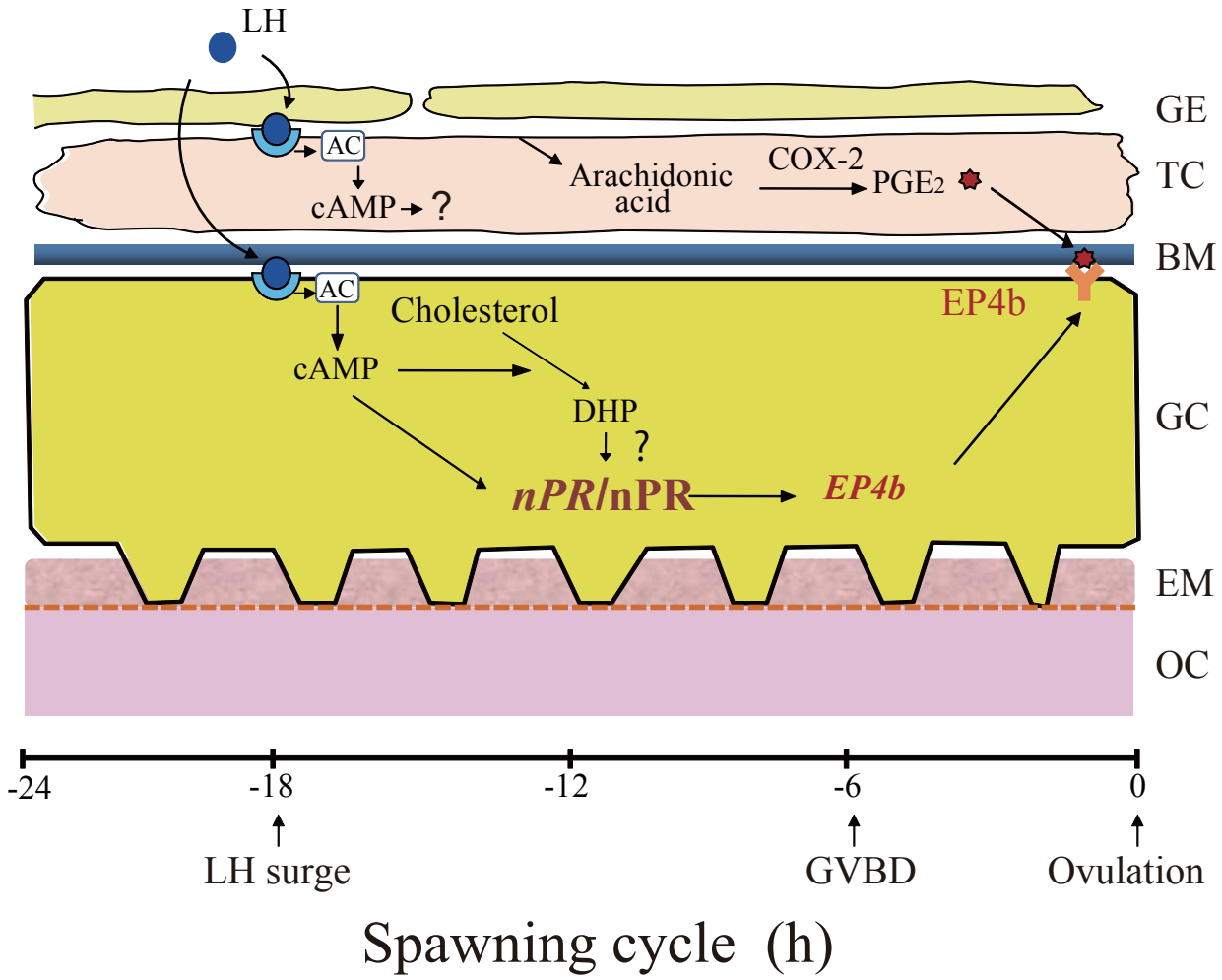

LH
AC
cAMP → ?
Arachidonic acid
COX-2
PGE2
GE
TC
BM
AC
Cholesterol
EP4b
cAMP
DHP
?
nPR/nPR
EP4b
GC
EM
OC
-24
-18
-12
-6
0
LH surge
GVBD
Ovulation
Spawning cycle (h)

Figure 8. A proposed model for EP4b expression in the GCs of medaka preovulatory follicles.

Time schedule of nPR-involving EP4b expression in the follicle layer of the preovulatory follicle is shown. GE, germinal epithelium; TC, theca cell; BM, basement membrane; GC, granulosa cell; EM, egg membrane; OC, oocyte; DHP, 17α, 20β-dihydroxy-4-pregnen-3-one; AC, adenylyl cyclase; COX-2, cyclooxygenase 2. For details, see the text.

Chapter 2

Expression of membrane progestin receptors in granulosa cells of medaka preovulatory follicles and their possible involvement in ovulation

ABSTRACT

Ovarian expression of membrane progestin receptor (mPR) mRNA was investigated using the teleost medaka. In the preovulatory follicles, transcripts of *mPRβ* were expressed in the follicles at lower levels while those of *mPRα* and *mPRγ* were dominantly expressed. *mPRα* and *mPRγ* were also expressed in the follicle granulosa cells (GCs). mRNA expression levels of mPRs in the follicles remained relatively constant during 24-h spawning cycles. *In vitro* analysis using HEK293T cells expressing medaka mPRα demonstrated that forskolin-elevated intracellular cAMP levels were lowered by treatment of the cells with the ligand 17α, 20β-dihydroxy-4-pregnen-3-one (DHP). Treatment of preovulatory follicles with forskolin (FSK) decreased the rates of ovulation and oocyte maturation. Further, expression of prostaglandin E_2 receptor subtype *EP4b* mRNA, but not classical nuclear progestin receptor (*nPR*) mRNA, in the follicle GCs was significantly inhibited by the same treatment. These results suggest that mPRα may play a role in the nPR-mediated expression of *EP4b* in the follicle GCs destined to ovulate.

INTRODUCTION

Progestins are known to be essential to reproduction in both male and female vertebrates. In mammals, the steroids trigger a series of events that lead to ovulation in the ovaries [Lipner and Wendelken, 1971; Baranczuk and Fainstat, 1976; Snyder et al., 1984; Robker et al., 2000; Conneely et al., 2002; Russell and Robker, 2007; Robker et al., 2009]. Progestins also promote final oocyte maturation and ovulation in non-mammalian vertebrates, such as amphibians [Josefsberg et al., 2007] and teleosts [Zhu et al., 2003a; Thomas et al., 2004; Tokumoto et al., 2006; Nagahama and Yamashita, 2008]. In addition, progestins play important roles in the functions of the testes, including stimulation of sperm motility in various vertebrate species [Ho and Suarez; 2001; Luconi et al., 2004; Thomas and Doughty, 2004; Thomas et al., 2009]. Previous studies revealed that these effects are mediated either by the classical nPR or by nonclassical mPRs [Nagahama and Yamashita, 2008; Thomas, 2012; Wang et al., 2014].

We previously reported that prostaglandin E_2 signaling is required for successful ovulation in the teleost medaka [Fujimori et al., 2011; Fujimori et al., 2012]. Our studies also demonstrated that expression of a prostaglandin E_2 receptor subtype, *EP4b*, but not its ligand-producing enzyme *COX-2*, was dramatically induced in the preovulatory follicles near the time of ovulation initiation, suggesting a mechanism by which the prostaglandin effect on medaka ovulation is regulated by *de novo* synthesis of the EP4b receptor protein. Consistent with this idea is our previous finding [Fujimori et al., 2012] that COX-2 activities and PGE_2 levels were fairly constant in the preovulatory follicles that were destined to ovulate. More recently, medaka ovulation was shown to be triggered by exposing the preovulatory follicles to LH approximately 17 h before

ovulation during the 24-h spawning cycle [Ogiwara et al., 2013] and that the synthesis of nPR preceded the induction of *EP4b* mRNA in the follicle GCs that had undergone LH surge [Chapter 1]. My detailed study revealed that expression of *EP4b* mRNA was under the control of the transcription factor nPR, which is activated by binding to the ligand DHP, a naturally occurring maturation-inducing hormone (MIH) in the medaka [Sakai et al., 1988].

In my previous attempts to localize *nPR* mRNA in the medaka ovary, I happened to observe the expression of *mPRα* mRNA in the follicle. Further detailed examinations revealed that not only *mPRα* but also *mPRγ* were expressed at substantial levels in preovulatory follicle cells. Therefore, it is reasonable to assume that the nuclear and membrane-bound progestin receptors associated with follicle cells would be simultaneously activated when intrafollicular levels of DHP are elevated after an LH surge. In the literature, there are no studies addressing the roles of mPRs in the medaka ovulatory process. In this chapter, I conducted experiments to examine whether mPRs expressed in the follicle cells might be involved in medaka ovulation.

MATERIALS AND METHODS

Animals and tissues

Adult orange-red medaka variants (*Oryzias latipes*) were purchased from a local supplier and maintained in indoor tanks under artificial reproductive conditions (10-h dark/14-h light cycles; temperature, 27°C). Ovaries and other tissues were removed at the indicated time points after a 24 h-spawning cycle was established. Isolation of preovulatory follicles and preparations of follicle layers and oocytes from the isolated follicles were conducted as previously described [Ogiwara et al., 2013]. Follicle staging

was conducted as previously reported [Iwamatsu et al., 1988]. All experimental procedures used in this chapter were approved by The Committee of the Center for Experimental Plants and Animals at Hokkaido University.

Preovulatory follicle culture

Preovulatory follicles (approximately 0.8 mm in diameter, postvitellogenic phase, stage IX) were isolated from the ovaries of three to five fish at 22 h before ovulation and pooled. Approximately 20 follicles were used per culture. Follicle cultures were conducted in 90% medium 199 solution (Eagle's medium 199; Dainippon-Sumitomo Seiyaku, Osaka, Japan), pH 7.4, containing 50 μM gentamycin in the absence or presence of medaka rLH as previously described in chapter 1. Appropriate concentrations of FSK were also added to the cultures (1 and 25 μM). After incubating for the time periods indicated, the follicles were assayed for oocyte maturation and ovulation. In some experiments, the follicles were collected and transcript expression levels of *mPRα, mPRγ, nPR,* or *EP4b* were determined by real-time RT-PCR. FSK was purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of GCs from spawning female medaka ovaries

Preovulatory follicles were collected from the ovaries of spawning female medaka at 3 h before ovulation as previously described [Ogiwara et al., 2013]. In brief, the follicle layers consisting of both GCs and TCs were separated from the oocytes using forceps. After washing three times with PBS, the tissues were placed in PBS containing 1 mM EDTA and 0.25% trypsin followed by gentle rotation for 30 min at room temperature. The treated samples were collected by centrifugation at 2000 rpm for 3

min. After three washes with 90% medium 199 solution, the precipitates containing GCs and TCs were suspended in the same medium and filtered with a 100-μm nylon filter (BD Bioscience, Bedford, MA). The resultant filtrates were cultured in the medium containing 50 μM gentamycin and 5% carp serum. After 48 h of culture, unattached cells were removed by gentle washing with PBS and the cells remaining attached to the dish were collected. Total RNA for RT-PCR analysis was isolated from the collected cells.

RT-PCR and real-time RT-PCR

Total RNA was separately prepared from various tissues of adult medaka using Isogen (Nippon Gene, Tokyo, Japan). RNA aliquots (2.5 μg) were reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with Oligo$(dT)_{20}$ Primer (Invitrogen) according to the manufacturer's instructions. The complimentary DNA (cDNA) prepared as above was used for PCR and real-time PCR. The cDNAs were amplified by PCR using the TaKaRa Ex Taq® Hot Start Version (Takara, Tokyo, Japan) as previously described [Chapter 1]. Real-time RT-PCR analyses were conducted using an ABI 7300 Real-Time PCR System (Life Technologies, Inc., Rockville, MD). The amplification efficiency was verified using the same system. The PCR reaction and data analysis were performed according to the procedures described in chapter 1. The gene transcript levels in the tissues were normalized using *actb* or ribosomal protein L7 (*rpl7*). Primers used for RT-PCR and real-time RT-PCR are listed in Table 2.

GloSensor cAMP assay of mPRs

Coding regions of mPRα and mPRγ were amplified by PCR with the fish ovary cDNA using KOD DNA polymerase (Toyobo, Tokyo, Japan). The amplified products were digested with *Eco*RI and *Xho*I and then gel-purified. The purified fragments were ligated into a pCMV vector that had previously been digested with the same enzymes. The sequences of the resulting vectors, pCMV-mPRα or pCMV-mPRγ, were confirmed by sequencing.

HEK293T cells (Riken cell bank, Tukuba, Japan) were cultured at 37ºC in DMEM (Wako) supplemented with 10% FBS (Wako), 1X penicillin-streptomycin-amphotericin B suspension (Wako), and 2 mM L-glutamine (Wako). The cells were transfected with Glosensor^{TM}-22F cAMP plasmid (Promega, Madison, WI) expressing a modified form of luciferase, which contains a cAMP binding motif, and pCMV-mPRα or pCMV-mPRγ using lipofectamine® 2000 (Invitrogen) in Opti-MEM medium (Invitrogen) according to the manufacturer's protocol. One day before transfection, 1-4×10^5 cells/ml were cultured on a 12-well plate. After 48 h of incubation, the medium was removed and replaced with fresh medium containing 1% Glosensor^{TM} cAMP reagent (Promega) and the cells were incubated for 2 h at room temperature with gentle shaking. The cells were then harvested by pipetting and re-suspended in 500 µl PBS containing 1 µM FSK. The luciferase activity in the cells was monitored using a luminometer. After 3 min, DHP (Sigma-Aldrich) was added at 1 µM to the suspension and the activities were further assessed every minute for 10 minutes.

Statistical analysis

All experiments were conducted at least three times to confirm the reproducibility of the results. The data are presented at the mean ± S.E.M. Data were analyzed using

Student's *t*-test, and $P<0.05$ was considered statistically significant.

RESULTS

mRNA expression levels of mPRs in various medaka tissues

Three mPR genes (mPRα, mPRβ and mPRγ) were identified in medaka using a search program from the NCBI (http://www.ncbi.nlm.nih.gov/). Preliminary RT-PCR analysis using total RNA isolated from spawning medaka ovaries detected transcripts of *mPRα* and *mPRγ*, but not *mPRβ.* This observation was further substantiated by real-time RT-PCR analysis (Fig. 9A). *mPRγ* transcripts were most abundant among the genes analyzed in the fish ovary. *mPRα* mRNA was also detected, but its abundance was approximately 1/3 that of *mPRγ*. Expression of *mPRβ* mRNA was very low compared with that of *mPRα* or *mPRγ*. Based on this finding, further studies were conducted only investigating mPRα and mPRγ.

mPRα and *mPRγ* mRNAs were both expressed in fish ovaries and testes (Fig. 9B and 9C). The tissue distribution pattern of medaka *mPRα* mRNA was very similar to that reported for spotted sea trout where *mPRα* was expressed only in reproductive and neuroendocrine tissues [Zhu et al., 2003a]. A wider tissue distribution of *mPRα* mRNA expression has been reported for other organisms, such as the goldfish [Tokumoto et al., 2006]. The results described above suggest that mPRα and mPRγ may play a role in medaka reproductive organs and brain.

Expression of mPRα and mPRγ mRNA in the fish ovary

Changes in the expression levels of *mPRα* and *mPRγ* mRNA were examined using whole ovaries of sexually matured female medaka with established 24 h-spawning

cycles by real-time RT-PCR. *mPRα* and *mPRγ* mRNAs were expressed at relatively constant levels through the spawning cycle (Fig. 10A and 10B). Transcripts of the two membrane progestin receptors were also analyzed using the preovulatory follicles that were destined to ovulate (Fig. 11A and 11B). Levels of *mPRα* transcripts did not change during the spawning cycle (Fig. 11A). In contrast, expression of *mPRγ* transcripts was low during the early postvitellogenic stage (-23 h) but elevated thereafter (-19 h ~ -3 h) (Fig. 11B). These results indicate that both *mPRα* and *mPRγ* transcripts were expressed at detectable levels in the fish ovary preovulatory follicles.

To gain further insight into the expression of *mPRα* and *mPRγ* mRNA in the preovulatory follicle, I compared their expression levels among the follicle, the follicle layer and follicle layer-derived GCs. Total RNA fractions were prepared and used for real-time RT-PCR analysis of *mPRα* and *mPRγ* mRNA expression (Fig. 12). Amplification efficiencies in real-time RT-PCR for *mPRα* and *mPRγ* primer sets were comparable. Levels of *mPRγ* transcripts were greater than those of *mPRα* not only in the whole preovulatory follicle but also in the follicle layer (Fig. 12A and 12B). The expression of *mPRα* and *mPRγ* in the GC fraction isolated from the follicle layer via the culture method was determined by RT-PCR (Fig. 12C). Transcripts of *MT3-MMP* (a marker for oocytes), *gelatinase B* (a marker for GCs), c*ollagen type I α-chain* (a marker for TCs), and *actb* (a control house-keeping gene) were also determined as controls (Fig. 12C). Among the marker genes assessed, only *gelatinase B* transcripts were detected, indicating that GCs were reliably isolated. Using the GC fraction, *mPRα* and *mPRγ* transcripts were successfully amplified. By further real-time RT-PCR analysis using the same fraction, increased expression of *mPRγ* over *mPRα* was observed (Fig. 12D). These results clearly indicate that both progestin receptor mRNAs were expressed in the

GCs of preovulatory follicles.

Collectively, the above results suggest that mPRα and mPRγ may play a role in the processes associated with preovulatory follicle GCs that are predicted to ovulate.

Effect of rLH on the expression of mPR mRNAs in the preovulatory follicle

The preovulatory follicles that had not been exposed to LH *in vivo* were isolated from the ovaries 22 h before ovulation and incubated with or without rLH. Overall, levels of *mPRα* transcripts in the rLH-treated follicles were slightly higher than those in the rLH-untreated follicles. The average values at each time point were 2-6 times greater in the rLH-treated follicles compared with the untreated follicles, but these changes were not statistically significant (Fig. 13A). Similar *in vitro* experiments were conducted to determine follicular expression of *mPRγ* mRNA (Fig. 13B). rLH had no effect on the follicular expression of *mPRγ*, and these results indicate that *mPRα* and *mPRγ* mRNA expression may not be under control of LH.

In vitro characterization of medaka mPRα and mPRγ using HEK293T cells

To examine the initial event triggered by the activation of medaka mPRs, HEK293T cells transiently expressing medaka mPRα or mPRγ were prepared. FSK treatment of the cells expressing mPRα increased intracellular cAMP levels, and the cyclic nucleotide levels reached maximal levels within 3 min. FSK-elevated nucleotide levels gradually decreased (Fig. 14A). However, intracellular cAMP levels decreased immediately after DHP was added to the reaction.

In the cells expressing medaka mPRγ, DHP treatment was without effect on the FSK-elevated intracellular cAMP levels (Fig. 14B). As controls, experiments using

HEK293T cells that had not been previously transfected with any progestin receptor cDNA were conducted. Addition of DHP to the cells containing elevated intracellular cAMP did not result in an immediate decrease in nucleotide levels (data not shown), suggesting that progestin receptors intrinsic to HEK293T cells, if any, had no effect on the results of our experiments. The above results imply that binding of DHP to medaka mPRα, but not mPRγ, causes a decrease in intracellular cAMP levels.

In vitro effect of FSK treatment on oocyte maturation and ovulation in the preovulatory follicle

Using our *in vitro* follicle culture system, I examined the effect of FSK on oocyte maturation and ovulation. Preovulatory follicles isolated 13 h before ovulation were incubated in the presence or absence of FSK (Fig. 15A). Oocyte maturation was assessed by counting oocytes that had undergone GVBD after 9 h of incubation (Fig. 15B). In the presence of 1 μM of FSK, the rate of GVBD was approximately 90%, which was comparable to that of the control. Addition of 25 μM FSK to the culture drastically reduced the rate of oocyte maturation. The effect of FSK on follicle ovulation rates was also examined. *In vitro* follicle ovulation was determined at 16 h after the start of incubation (Fig. 15C). Follicle ovulation was suppressed to a considerable extent even at 1 μM FSK and inhibited completely at 25 μM FSK. These results indicate that ovulation was more sensitive to FSK than was oocyte maturation.

Effect of FSK on the expression of EP4b, an ovulation-related gene in the medaka

As observed in the previous section, follicle ovulation was significantly inhibited at 1 μM FSK while oocyte maturation was not affected by the same treatment. Ovulation is a

genomic event involving transcription of many genes in the follicle cells of the preovulatory follicles. This information together with the finding presented in Fig. 15 led me to hypothesize that 1 μM FSK treatment of the follicles may selectively disorder a well-regulated expression pattern of ovulation-related genes in the follicle cells. To test this hypothesis, I examined the effect of FSK on the expression of the *nPR* and *EP4b* genes, both of which have been identified as ovulation-related genes in medaka [Chapter 1]. Preovulatory follicles were isolated from spawning medaka ovaries 13 h before ovulation and incubated with 1 μM FSK, and expression levels of the two genes were determined by real-time RT-PCR (Fig. 16A). No difference was observed in *nPR* mRNA levels between FSK-treated and FSK-untreated follicles (Fig. 16B). However, expression of *EP4b* mRNA was significantly reduced when the follicles were incubated with FSK (Fig. 16C). These results indicate that an increased intracellular cAMP concentration induced by FSK treatment in the preovulatory follicle GCs was unfavorable for transcription of *EP4b* gene, but not for the *nPR* gene.

DISCUSSION

Since mPRα was first identified as a novel membrane-bound progestin receptor involved in meiotic maturation of the spotted sea trout [Zhu et al., 2003a], the existence of this receptor has been demonstrated in a variety of species including mammals [Zhu et al., 2003b; Thomas et al., 2007; Dressing et al., 2012]. However, no studies dealing with medaka mPRs have been reported to date. In the present study, I observed that oocytes of preovulatory follicles in the spawning female medaka ovary expressed *mPRα* (data not shown), which is consistent with data reported for other fish [Zhu et al., 2003a]. The current study also showed that preovulatory follicle GCs expressed both

mPRα and *mPRγ* transcripts.

mPRα mRNA levels were constant in the fish preovulatory follicles throughout the 24 h spawning cycle whereas *mPRγ* mRNA levels were slightly increased. Expression of *mPRα* and *mPRγ* mRNA did not increase in the follicles incubated with rLH *in vitro*. These findings are consistent with the idea that expression of the *mPRα* and *mPRγ* genes is probably regulated in an LH-independent manner in the fish preovulatory follicles. I tentatively presume that both membrane progestin receptors are constitutively expressed at least in the follicles that enter the post-vitellogenic stage (stages VIII and IX). As documented in previous studies by other investigators, ovarian follicles from spotted sea trout [Zhu et al., 2003a], Atlantic croaker [Tubbs et al., 2010] and goldfish [Tokumoto et al., 2006] showed increased mRNA and protein expression of mPRs following gonadotropin treatment.

It is well established that oocyte maturation in fish is triggered by the exposure of preovulatory follicles to LH. An LH surge results in the production of MIS, which is known as DHP in most teleost species [Nagahama and Yamashita, 2008]. The binding of DHP to mPR expressed on the cell surface membrane of the oocyte activates the Giα protein, which in turn reduces intracellular cAMP levels in the oocyte, eventually leading to the formation and activation of maturation-promoting factor (MPF) [Nagahama and Yamashita, 2008]. Activation of Giα and the subsequent reduction in intracellular cAMP levels upon the activation of mPRα by DHP was reported in previous studies [Nagahama and Yamashita, 2008]. In the medaka, a drastic rise in DHP levels in the preovulatory follicle occurs approximately 5 h after LH surge [Iwamatsu et al., 1988; Ogiwara et al., 2013]. As reported in Chapter 2, 25 μM FSK treatment had a strong inhibitory effect on the oocyte maturation rate. This finding strongly suggests the

involvements of mPRs expressed on the oocyte surface in the oocyte maturation in medaka preovulatory follicles. The expression of nPR is also induced by LH surge in the follicle GCs by the time that DHP becomes available in the follicles [Chapter 1]. The transcription factor, nPR, which is activated by associating with DHP, activates various genes necessary for ovulation. Because *mPRα* and *mPRγ* transcripts, and presumably their corresponding proteins, are expressed in the GCs of preovulatory follicles, it is reasonable to assume that, in addition to mPRα at the oocyte cell membranes, the two mPRs associated with the somatic cell membranes could be intermediaries for DHP.

What is the biological significance of GC mPR activation in the preovulatory follicles? As described above, a large number of ovulation-related genes are transcribed and translated in the follicles that are destined to ovulate. These processes, so-called genomic events, are known to take place in the follicle cells including GCs. Activation of mPRs might be necessary for a series of transcriptional and translational events associated with ovulation. In this context, an interesting result in this study was the effect of FSK treatment on the preovulatory follicles that had been exposed by LH *in vivo*. I observed differential effects of FSK on the preovulatory follicles, depending on the concentrations used. Only *in vitro* follicle ovulation was detectably inhibited at a low FSK concentration (1 μM) while the rates of both follicle ovulation and GVBD were strongly suppressed by a higher FSK concentration (25 μM). The requirement of higher FSK concentrations for the inhibition of GVBD was also reported in the previous study using brook trout as GVBD was inhibited by FSK at concentrations greater than 10 μM [DeManno and Goetz, 1987]. The present results of FSK effects on the medaka preovulatory follicles clearly indicate that ovulation is more sensitive to FSK treatment

than is oocyte maturation. I assumed that analyses of the follicles treated with 1 μM FSK rather than 25 μM FSK were more appropriate to understand the selective effects on the processes tightly associated with the follicle GCs. Treatment of preovulatory follicles with FSK at 1 μM caused a reduction in *EP4b* mRNA expression in the GCs. This finding suggests that FSK-elevated intracellular cAMP levels in the follicle cells is unfavorable for transcription and translation of the EP4b gene. It should be noted that medaka mPRα, but not mPRγ, caused a decrease in intracellular cAMP levels as demonstrated by the present *in vitro* experiments using HEK293T cells expressing medaka mPRs. From these considerations, I tentatively assume that mPRα could contribute to the reduction of intracellular cAMP in the preovulatory follicle GCs.

I have recently demonstrated that the transcription factor nPR plays an indispensable role in *EP4b* gene expression in the medaka preovulatory follicles [Chapter 1]. Expression of nPR occurs immediately after LH surge (approximately 17 h before ovulation) and reaches maximal levels within 6-8 h while transcription of the *EP4b* gene is delayed approximately 8-10 h behind nPR expression [Chapter 1; Fujimori et al., 2012]. In addition, I previously observed that both *nPR* and *EP4b* mRNA expression were induced by FSK alone when the preovulatory follicles had not yet been experienced an LH surge *in vivo* (Fig. 5). However, I observed a differential effect of FSK on *EP4b* and *nPR* expression in the follicles that had been exposed to LH *in vivo*. As shown in Fig. 15, FSK treatment of the follicles inhibited expression of *EP4b* mRNA while the treatment had no effect on *nPR* expression. These findings indicate that elevated intracellular cAMP levels may be favorable for the follicles to transcribe and translate the nPR gene only for 6-8 h after LH surge *in vivo*. Once the nPR protein is generated in the follicle, higher intracellular cAMP levels are physiologically

irrelevant to EP4b gene activation. Transcription of *Pai-1* and *MT2-MMP* and other ovulation-related genes/proteins were reported to be possibly regulated by LH-induced nPR in the medaka preovulatory follicle [Ogiwara et al., 2015; Ogiwara et al., 2013]. Among these, the expression of *Pai-1* was found to be affected by 1 μM FSK, while that of *MT2-MMP* was not (unpublished results). These observations lead us to speculate that reduced intracellular cAMP levels may be essential to the transcription of some, if not all, of the ovulation-related genes governed by the transcription factor nPR in the GCs of the follicles that were predicted to ovulate. At present, I have no explanation for how reduced intracellular cAMP levels in the GCs activate the *EP4b* gene.

It is interesting to note the recent finding that the mPRα protein is localized to the plasma membranes of both GCs and TCs isolated from Atlantic croaker ovaries [Dressing et al., 2010]. It was further demonstrated that endogenous mPR signaling in the ovarian follicle may suggest a role for mPRα in mediating the antiapoptotic actions of progestins in ovarian follicle cells. A similar physiological role for mPRα might be applicable in the medaka ovary. Because of difficulty in producing specific mPR antibodies, I failed to examine the protein expression of mPRs. I tentatively assumed the expression of mPR proteins is probably in parallel with that of the mPR mRNA. As suggested by the present study, mPRα and mPRγ proteins are presumed to localize to the plasma membranes of the GCs in the large preovulatory follicles of the medaka. Therefore, mPRα associated with the GC membranes of the follicles may have a dual role in progestin receptor activation when DHP is generated; one role is to facilitate nPR-mediated transcription of many ovulation-related genes and the other is to promote antiapoptotic activity in the cells. In the somatic cells of small-growing follicles, mPRs

may mediate antiapoptotic actions alone when the follicles are exposed to DHP, which is generated and diffused from large preovulatory follicles of spawning female medaka ovaries.

In this chapter, I assessed the expression of both *mPRα* and *mPRγ* mRNA using specific primers for the respective transcripts, although I previously described that *mPRα* was only one *mPR* mRNA species expressed in the fish ovary [Chapter 1]. This discrepancy was due to the use of less specific PCR primers in chapter 1. The current data on real-time RT-PCR undoubtedly showed the expression of the two distinct *mPRs* in the medaka ovarian follicles.

I also showed the expression of *mPRγ* in the preovulatory follicle GCs in the medaka ovary. Levels of *mPRγ* mRNA were greater than that of *mPRα.* Interestingly, mPRγ showed a property distinct from mPRα in the GloSensor cAMP assay using HEK293T cells; unlike mPRα, medaka mPRγ did not reduce intercellular cAMP levels. I tentatively assume that mPRγ may not be involved in the process of nPR-mediated *EP4b* transcription in the GCs. At present, the role of mPRγ in the follicles is not known.

In summary, the present study showed the expression of *mPRα* and *mPRγ* in the large preovulatory follicle GCs in the medaka. *In vitro* activation of mPRα by binding with the ligand DHP decreased intracellular cAMP concentrations in the HEK293T cells expressing medaka recombinant mPRα. It is highly possible that mPRα associated with the cell membranes of oocytes and mPRα present on the cell membranes of GCs could be simultaneously activated by DHP, the levels of which would be elevated in the follicle approximately 12 h before ovulation. My data suggest that, together with the classical nuclear progestin receptor nPR, GC membrane mPRα plays a role in

expression of prostaglandin E_2 receptor subtype *EP4b*. To our knowledge, this is the first study to determine mPRα expression in somatic cells of to elucidate the function of this family of proteins in the ovulatory process.

Table 2. Primer nucleotide sequences used in Chapter 2

Gene	Primer sequences, Forward (F) and Reverse (R) primer (5'-3')	Accession No.
For Real-time RT-PCR		
EP4b	F:CAGATGGTGATCCTGCTCAT	AB563504
EP4b	R:GCCAGGAGGTCTTCATTGAT	
mPRα	F:CCACGTTCTCTACGGCTACA	NM_001177476
mPRα	R:CCAATCTGCTCCATCACAAC	
mPRβ	F:GGAAGAGAGTCGGTCTGAGGTT	NM_001201495
mPRβ	R:CAAGGACGAGGTCTGAGATGTG	
mPRγ	F:CCGTCAAAAGATCAATCTGT	NM_001201493
mPRγ	R:CGCCCAATGCTCTCCATCAC	
nPR	F:AGAGGAACCCATCACCCTTC	AB854418
nPR	R:TGAGGACTCCATAGTGGCAA	
actb	F:TGACGGAGCGTGGCTACTC	D89627
actb	R:TCCTTGATGTCACGGACAATTT	
rpl7	F: CGCCAGATCTTCAACGGTGTAT	DQ118296
rpl7	R: AGGCTCAGCAATCCTCAGCAT	
For GloSensor cAMP assay		
mPRα	F:CCGGAATTCATGGCAACGGTTGTGATG	NM_001177476
mPRα	R:CCGCTCGAGTCACTCCTCTTTGTCGTG	
mPRγ	F:CCGGAATTCATGGCCACCATTGTGATG	NM_001201493
mPRγ	R:CCGCTCGAGTCATTTGGATTTACTTTG	
For RT-PCR		
mPRα	F:TGCAAGTGCGTGAACCGCAGC	NM_001177476
mPRα	R:ATGTGACGTGATCGATGACTA	
mPRγ	F:CGGCTCCTACCTGGATTACGT	NM_001201493
mPRγ	R:CTTGTATGTTCAGCAGGCCGA	
MT3-MMP	F:AAGTCATGCAGTCTGCTATTGCTG	AB072929
MT3-MMP	R:TGGATCGTTGGAGTGTTCAAGAC	
gelatinase B	F: CAAAACAGATCCTAAACCAACTGT	AB033755
gelatinase B	R: ATTTTAGGAGATCATATTTCACGT	
Collagen type I α1chain	F: GAGAAATCTGGACTTGAA	AB 280535
Collagen type I α1chain	R: GTACAGAGCAACCGAGTT	
actb	F:CAGACACGTATTTGCCTCTG	D89627
actb	R:CAAGTCGGAACACATGTGCA	

Figure 9

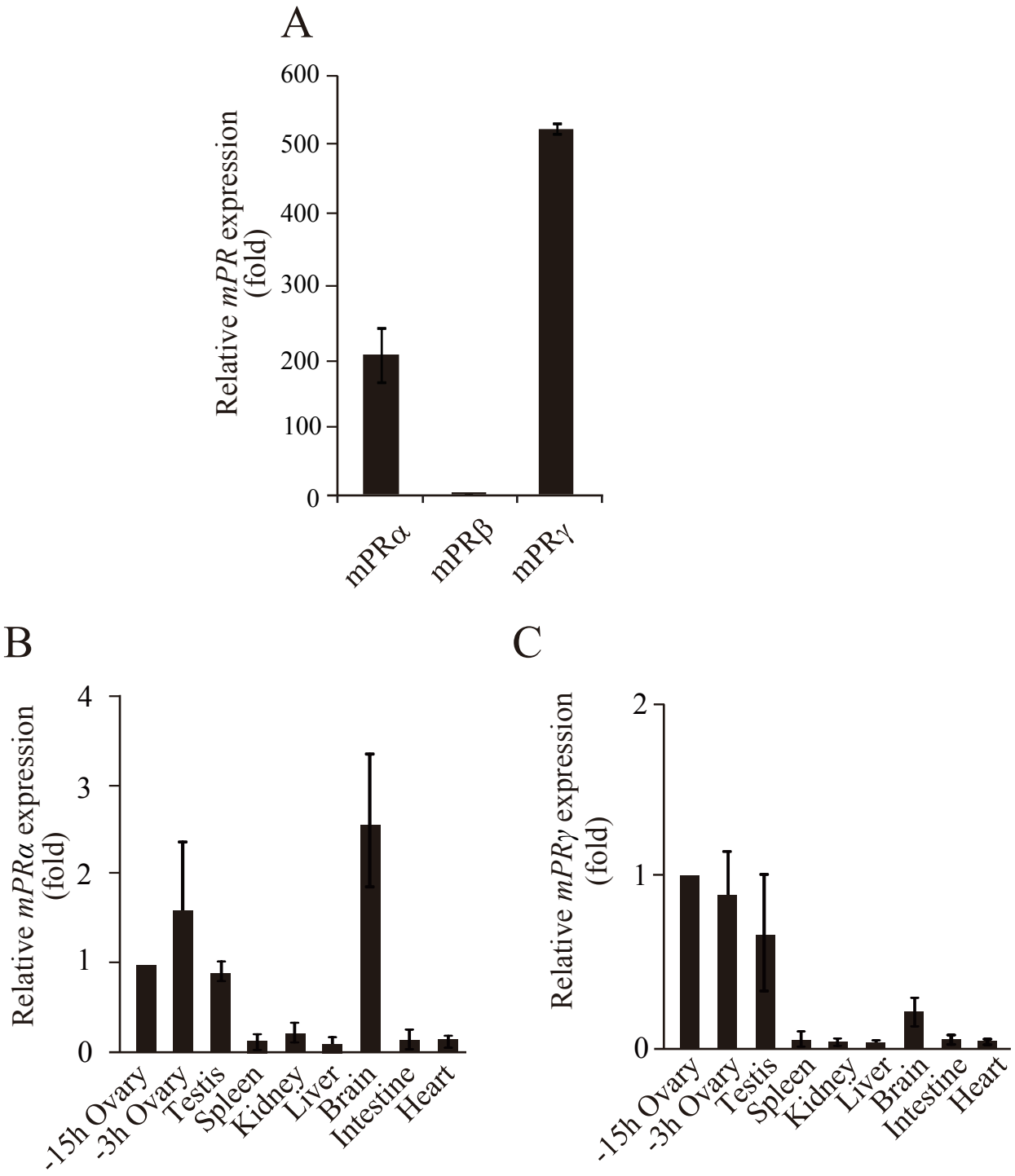

A
Relative mPR expression (fold)
600
500
400
300
200
100
0
mPRα
mPRβ
mPRγ
B
Relative mPRα expression (fold)
4
3
2
1
0
-15h Ovary
-3h Ovary
Testis
Spleen
Kidney
Liver
Brain
Intestine
Heart
C
Relative mPRγ expression (fold)
2
1
0
-15h Ovary
-3h Ovary
Testis
Spleen
Kidney
Liver
Brain
Intestine
Heart

Figure 9. mRNA expression levels of *mPRs* in various medaka tissues

(A) Expression levels of *mPRα*, *mPRβ*, and *mPRγ* transcripts were determined by real-time RT-PCR using total RNA isolated from the ovaries of spawning female medaka at 15 h before ovulation. The expression levels were normalized to those of *actb* and are presented as the fold change relative to *mPRβ* transcripts. The results are the mean ± S.E.M (n=5). (B) Expression levels of *mPRα* transcripts were determined by real-time RT-PCR using total RNA isolated from various tissues at the intermediate point (12 h before ovulation) of the 24 h-spawning cycle with the exception of the ovaries, which were obtained at 15 and 3 h before ovulation. The expression levels were normalized to those of *rpl7* and presented as the fold change relative to the level of each gene in the -15 h ovary. The results are the mean ± S.E.M (n=5). (C) Expression levels of *mPRγ* transcripts were determined as in (B). The results are the mean ± S.E.M (n=5).

Figure 10

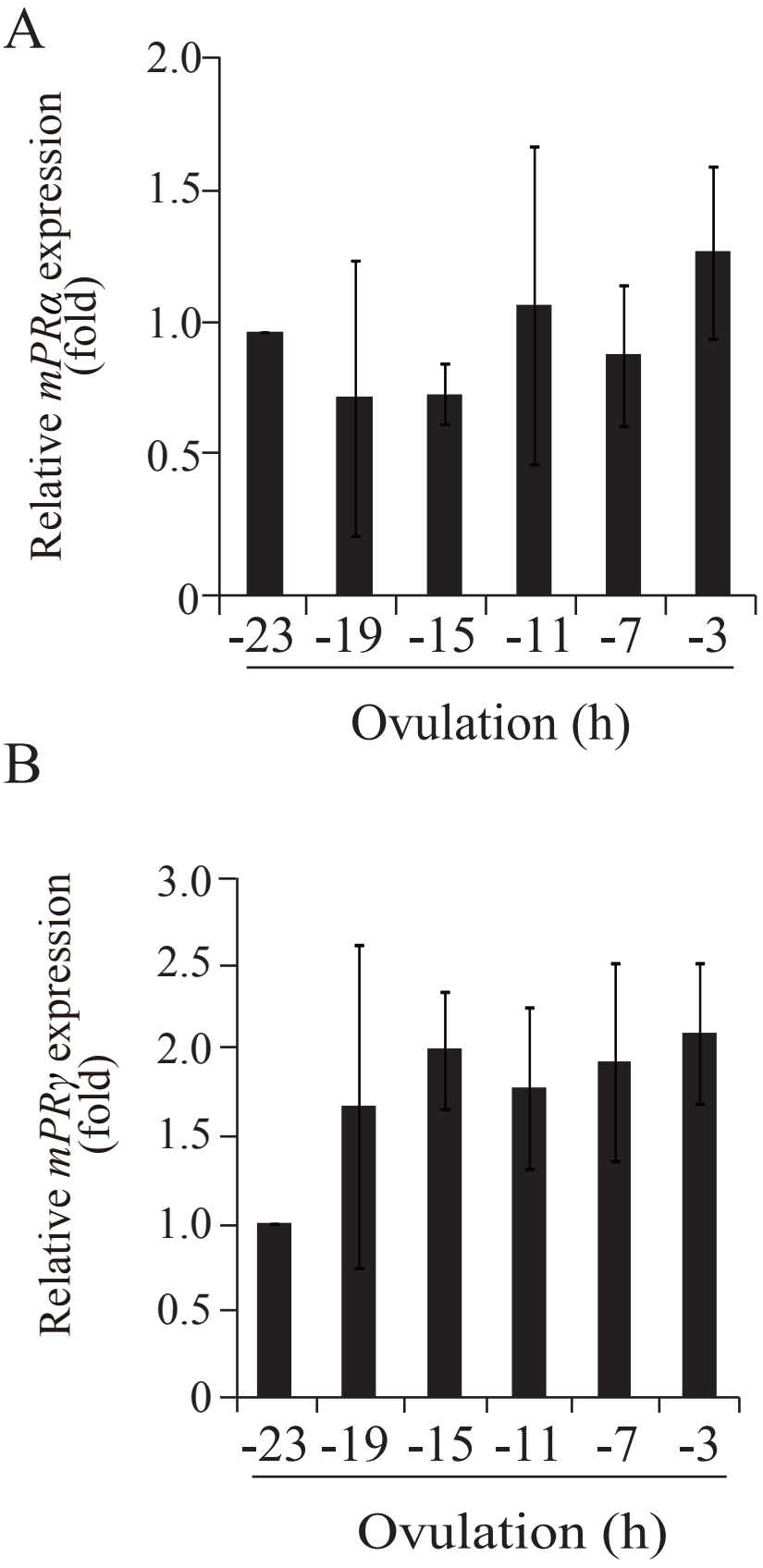
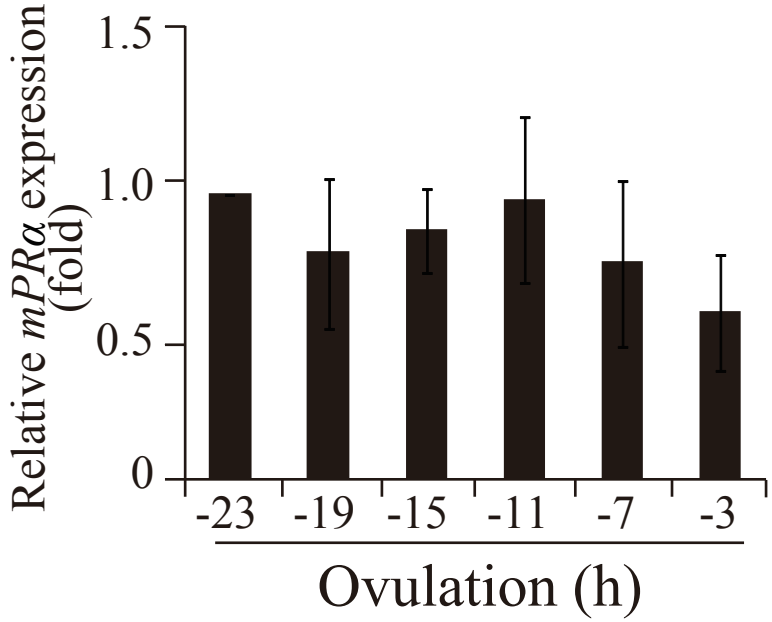

A
Relative mPRα expression (fold)
2.0
1.5
1.0
0.5
0
-23 -19 -15 -11 -7 -3
Ovulation (h)
B
Relative mPRγ expression (fold)
3.0
2.5
2.0
1.5
1.0
0.5
0
-23 -19 -15 -11 -7 -3
Ovulation (h)

Figure 10. Expression of *mPRα* and *mPRγ* mRNAs in the fish ovary

(A) Real-time RT-PCR analysis was performed for *mPRα* using total RNA isolated at the indicated time points from the ovaries of adult medaka with 24-h spawning cycles. The expression levels of *mPRα* were normalized to the levels of *actb* transcript. Relative *mPRα* expression levels are expressed as the fold change in mRNA levels relative to expression levels in the -23 h ovary. The results are the mean ± S.E.M (n=5). (B) Real-time RT-PCR analysis for *mPRγ* was performed as in (A). The results are the mean ± S.E.M (n=5).

Figure 11

A


Relative mPRα expression (fold)
1.5
1.0
0.5
0
-23
-19
-15
-11
-7
-3
Ovulation (h)

B

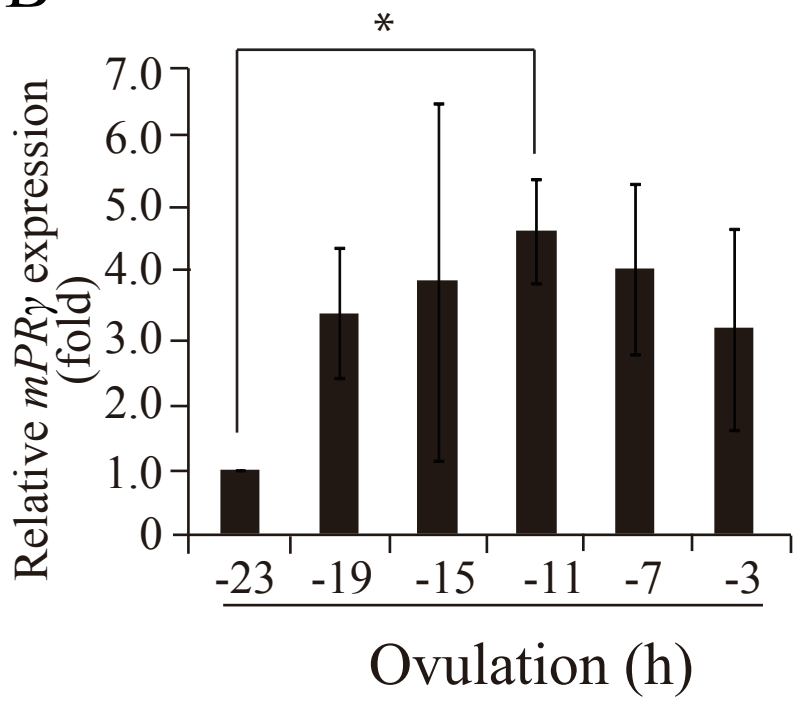
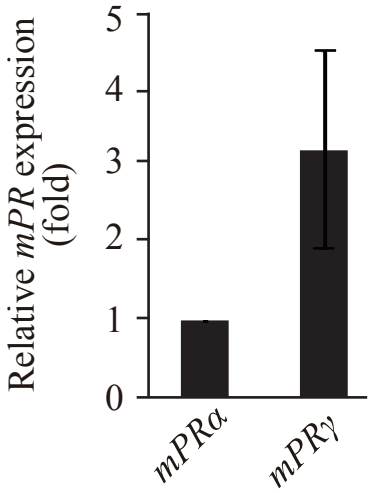

*
Relative mPRγ expression (fold)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
-23
-19
-15
-11
-7
-3
Ovulation (h)

Figure 11. Expression of *mPRα* and *mPRγ* mRNAs in the fish preovulatory follicle

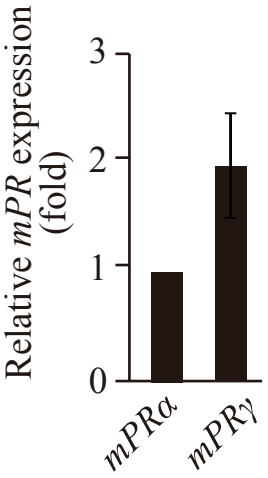
(A) Real-time RT-PCR analysis was performed for *mPRα* using total RNA isolated at the indicated time points from the preovulatory follicles of adult medaka with 24-h spawning cycles. The expression levels of *mPRα* were normalized to the levels of *actb* transcript. Relative *mPRα* expression levels are expressed as the fold change in mRNA levels relative to those in the -23 h follicle. The results are the mean ± S.E.M (n=5). (B) Real-time RT-PCR analysis was performed for *mPRγ* as in (A). The results are the mean ± S.E.M (n=3). The asterisk denotes significant differences ($P<0.05$).

Figure 12

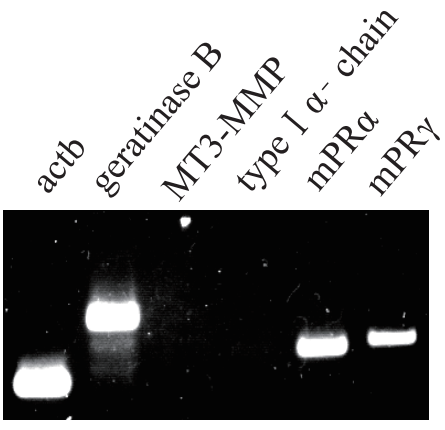
A



B



C



D

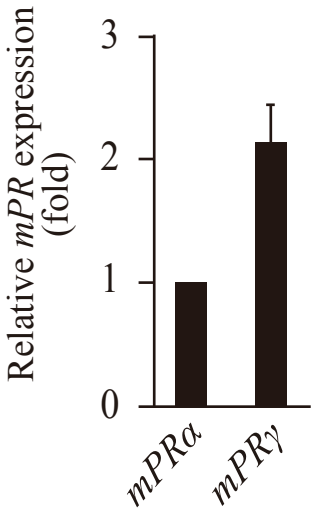


Figure 12. Expression of *mPRα* and *mPRγ* mRNAs in the preovulatory follicle GCs

(A) Real-time RT-PCR analysis was performed for *mPRα* and *mPRγ* using total RNA isolated 7 h before ovulation from the preovulatory follicles of adult medaka with 24-h spawning cycles. The expression levels were normalized to the levels of *actb* transcript, and relative expression levels are expressed. The results are the mean ± S.E.M (n=5). (B) Real-time RT-PCR analysis was performed for *mPRα* and *mPRγ* using total RNA isolated from the follicle layers of preovulatory follicles at 7 h before ovulation. Normalization and presentation of the results (n=5) were as in (A). (C) RT-PCR analysis was performed using total RNA isolated from the preovulatory follicle GCs using our culture method. Transcripts of *mPRα* and m*PRγ* were detected. Transcripts of *MT3-MMP* (a marker for oocytes), *gelatinase B* (a marker for GCs), *collagen type I α-chain* (a marker for TCs) and *actb* (a control house-keeping gene) were also determined for comparison. (D) Real-time RT-PCR analysis was performed for *mPRα* and *mPRγ* using the same total RNA as in (C). Normalization and presentation of the results (n=4) were as in (A).

Figure 13

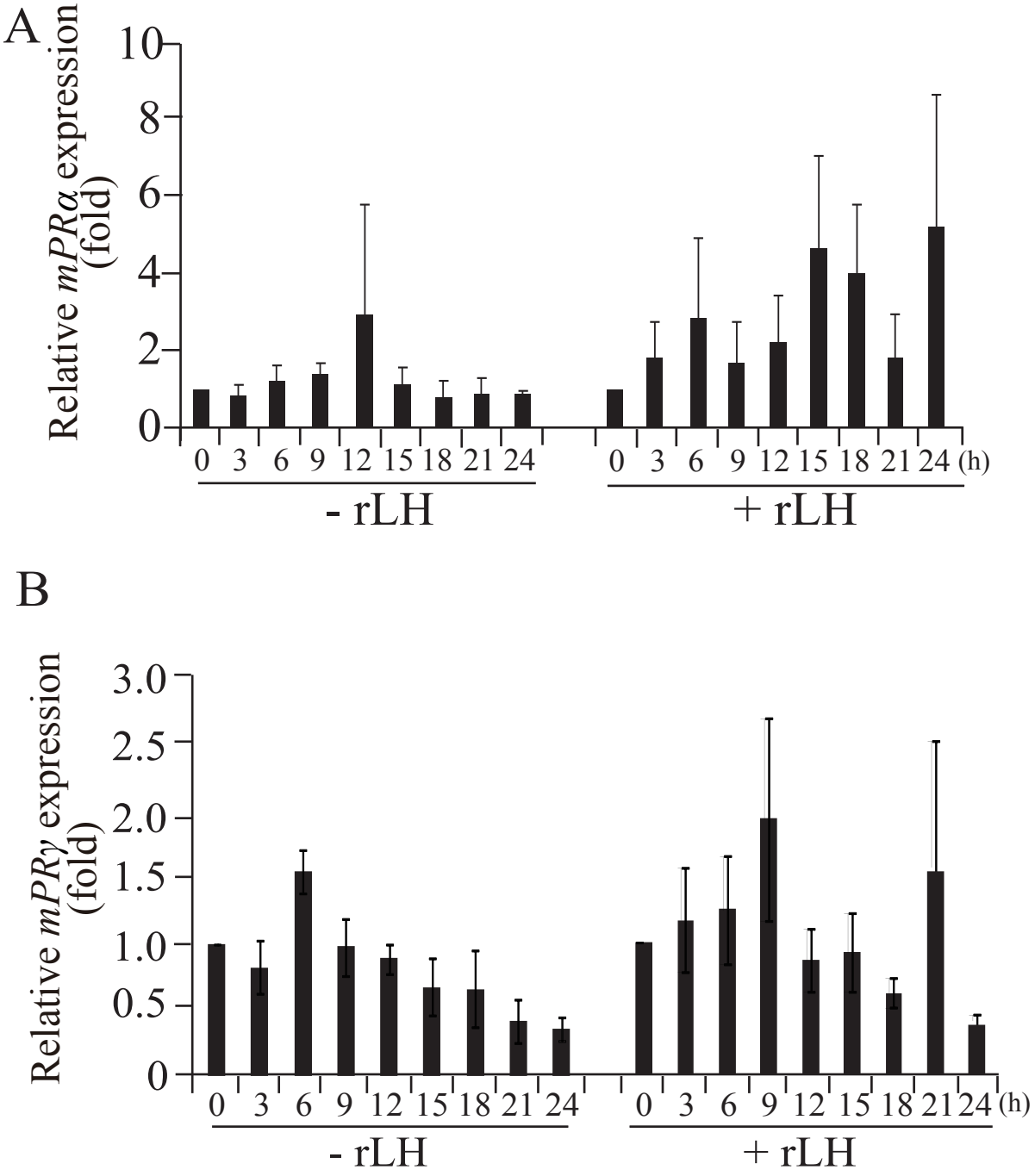

A
Relative mPRα expression (fold)
10
8
6
4
2
0
0 3 6 9 12 15 18 21 24
0 3 6 9 12 15 18 21 24 (h)
- rLH
+ rLH
B
Relative mPRγ expression (fold)
3.0
2.5
2.0
1.5
1.0
0.5
0
0 3 6 9 12 15 18 21 24
0 3 6 9 12 15 18 21 24 (h)
- rLH
+ rLH

Figure 13. Effect of rLH on the expression of *mPR* mRNA in the preovulatory follicle

(A) Preovulatory follicles were isolated from spawning medaka ovaries 22 h before ovulation and incubated with medaka rLH (100 μg/ml). Total RNA was isolated from the *in vitro* incubated follicles obtained at the indicated time points and used for real-time RT-PCR analysis of *mPRα* mRNA. The expression levels were normalized to that of *actb* and expressed as the fold change compared to expression levels in the -22 h follicles. The results are presented as the mean ± S.E.M (n=4). (B) Real-time RT-PCR analysis was conducted for *mPRγ* expression. Treatment of the follicles with rLH, total RNA isolation, and normalization and presentation of the data were as in (A). The results are presented as the mean ± S.E.M (n=4).

Figure 14

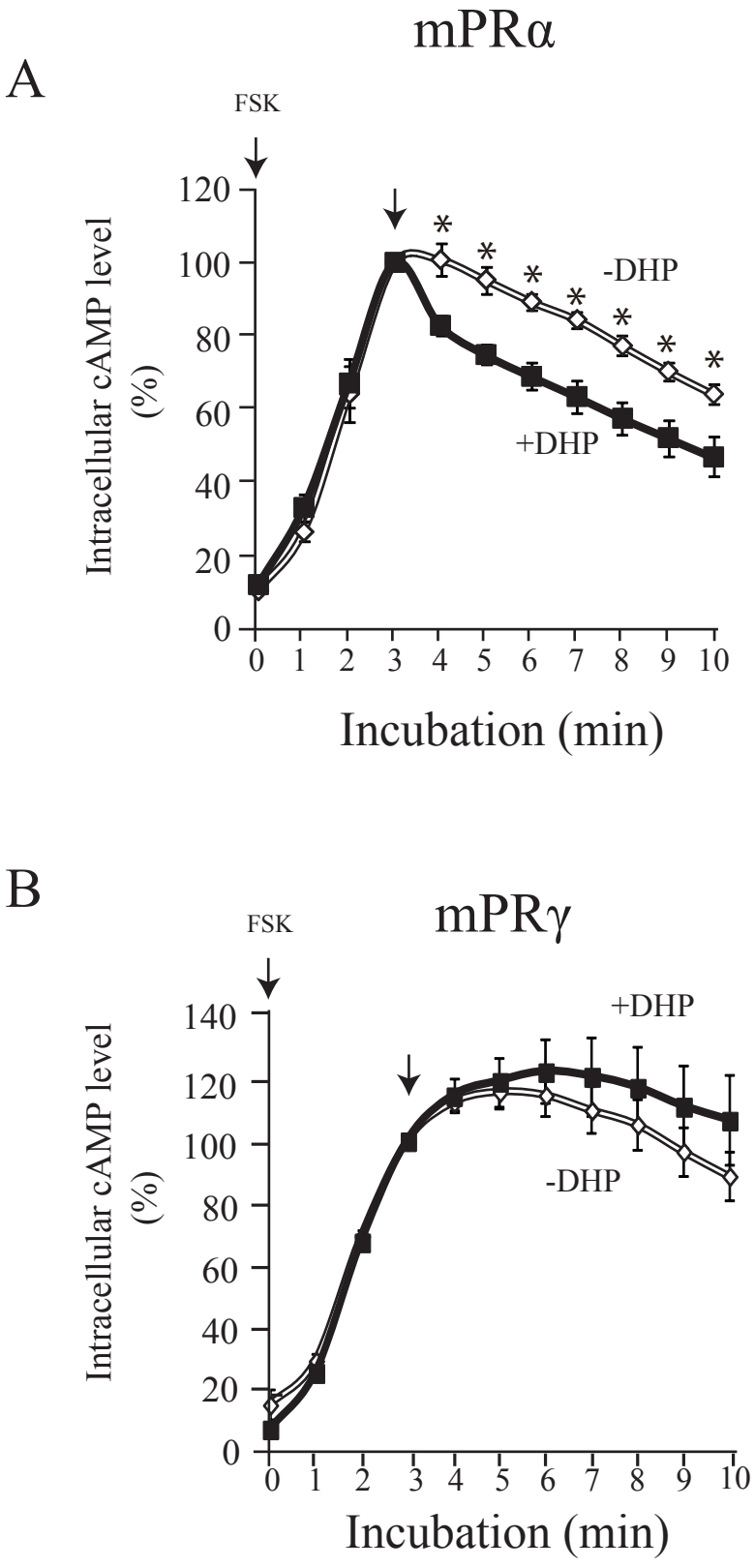

A
mPRα
FSK
Intracellular cAMP level (%)
120
100
80
60
40
20
0
-DHP
+DHP
0 1 2 3 4 5 6 7 8 9 10
Incubation (min)
B
mPRγ
FSK
Intracellular cAMP level (%)
140
120
100
80
60
40
20
0
+DHP
-DHP
0 1 2 3 4 5 6 7 8 9 10
Incubation (min)

Figure 14. *In vitro* characterization of medaka mPRα and mPRγ using HEK293T cells

(A) HEK293T cells transfected with Glosensor^{TM}-22F cAMP plasmid and pCMV-mPRα were treated with FSK (1 μM) at 37°C in PBS. After 3 min of incubation with FSK, the cells were further incubated in the presence or absence of DHP (1 μM) for 7 min. Luciferase activities of the cells, which represented intracellular cAMP levels, were monitored every minute through the incubation. The maximal enzyme activity value at 3 min after the start of FSK incubation was set at 100%. Relative levels of cAMP are shown. The results are presented as the mean ± S.E.M (n=4). Asterisks denote significant differences at $P<0.05$. (B) HEK293T cells transfected with Glosensor^{TM}-22F cAMP plasmid and pCMV-mPRγ were used. Treatment of the cells with FSK and subsequently with DHP was as described in (A). Luciferase activities of the cells were monitored, and the results (n=4) are shown.

Figure 15

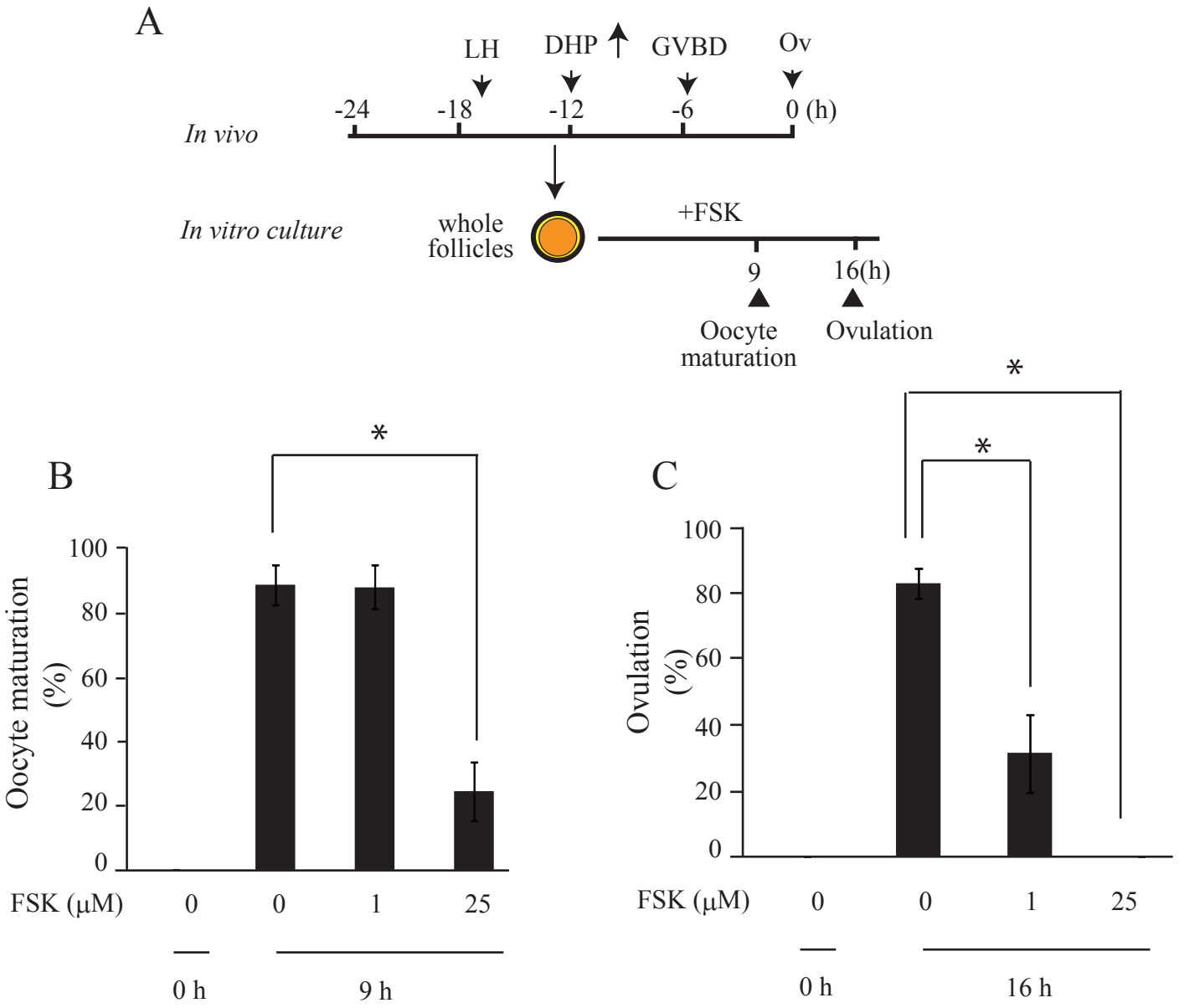

A
LH
DHP
GVBD
Ov
-24
-18
-12
-6
0 (h)
In vivo
In vitro culture
whole follicles
+FSK
9
16(h)
Oocyte maturation
Ovulation
B
Oocyte maturation (%)
100
80
60
40
20
0
FSK (μM)
0
0
1
25
0 h
9 h
*
C
Ovulation (%)
100
80
60
40
20
0
FSK (μM)
0
0
1
25
0 h
16 h
*
*

Figure 15. *In vitro* effects of FSK treatment on oocyte maturation and ovulation of the preovulatory follicle

(A) Preovulatory follicles that were destined to ovulate were isolated 13 h before ovulation and incubated with or without FSK. Rates of oocyte maturation (i.e., GVBD) and follicle ovulation were determined at 9 h and 16 h, respectively, after the start of incubation. (B) The number of the follicles that had undergone GVBD was assessed at 0 or 9 h after incubating with FSK or ethanol. FSK concentrations tested were 0 (ethanol only), 1 and 25 μM. The rates of oocyte maturation relative to that of follicles without FSK or ethanol are shown. The results are presented as the mean ± S.E.M (n=4). (C) The number of the follicles that had undergone ovulation was determined at 0 or 16 h after incubating with FSK or ethanol. FSK concentrations tested were the same as in (B). The rates of ovulation relative to that of follicles incubated with no substances are shown. The results are presented as the mean ± S.E.M (n=4). Asterisks denote significant differences at $P<0.05$.

Figure 16

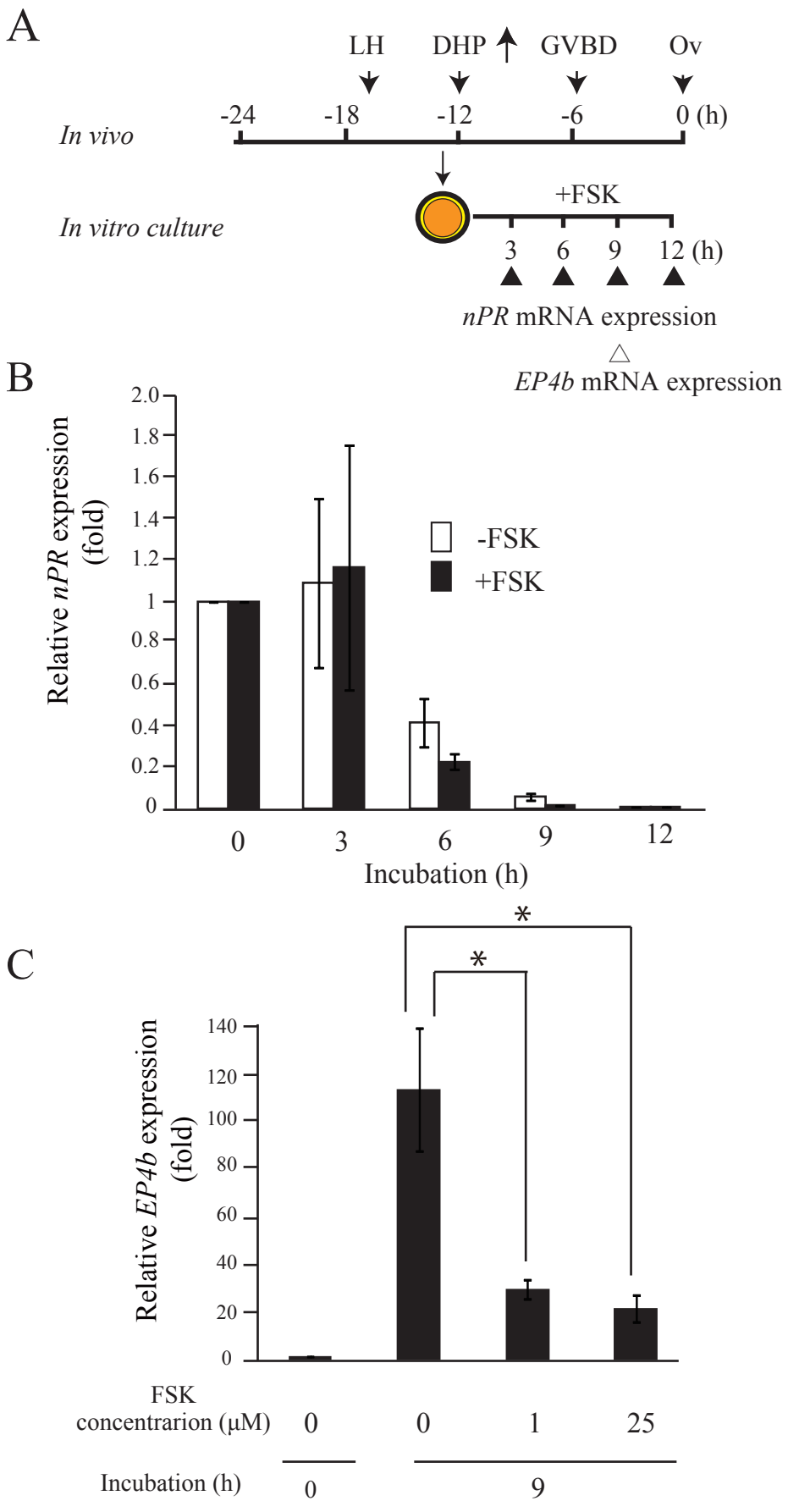

A
LH
DHP
GVBD
Ov
-24
-18
-12
-6
0 (h)
In vivo
In vitro culture
+FSK
3
6
9
12 (h)
nPR mRNA expression
△
EP4b mRNA expression
B
Relative nPR expression (fold)
2.0
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-FSK
+FSK
0
3
6
9
12
Incubation (h)
C
*
*
Relative EP4b expression (fold)
140
120
100
80
60
40
20
0
FSK concentrarion (μM)
0
0
1
25
Incubation (h)
0
9

Figure 16. *In vitro* effects of FSK treatment on *EP4b* and *nPR* mRNA expression in the preovulatory follicle

(A) Preovulatory follicles were isolated at 13 h before ovulation and incubated with or without FSK. Expression levels of the two genes were analyzed by real-time RT-PCR. *nPR* expression was analyzed every 3 h (▲), and *EP4b* was analyzed 9 h after incubation (△). (B) Total RNA was isolated from the *in vitro* incubated follicles taken at the black triangle (▲) indicated time points and used for real-time RT-PCR analysis of *EP4b* mRNA. The expression levels were normalized to that of *actb* and expressed as the fold change compared to the expression levels in the 0 h follicles. The results are presented as the mean ± SEM (n=3~5). Asterisks denote significant differences at $P<0.05$. (C) Real-time RT-PCR analysis was conducted for *nPR* expression. Total RNA isolation, normalization and presentation of the data were as in (B). The results are presented as the mean ± S.E.M (n=4).

GENERAL DISCUSSION

In this study, I investigated the molecular mechanisms regulating expression of the PGE_2 receptor subtype EP4b, which plays a critical role in the ovulation of the teleost medaka.

EP4b is expressed in the preovulatory follicle GCs at very low levels during the first half of the 24-h spawning cycle, but its expression level is dramatically elevated in the second half of the spawning cycle [Fujomori et al., 2011; Fujimori et al., 2012]. My study demonstrated that dramatic increases in *EP4b* mRNA expression in the follicle were triggered by LH treatment *in vitro*. This finding, together with the fact that *in vitro* follicle ovulation could be completely suppressed by an EP4b receptor antagonist (GW627368), indicates that EP4b is undoubtedly an ovulation-related gene/protein in medaka [Fujimori et al., 2012]. To my knowledge, there have been no previous studies investigating genes/proteins that are critically involved in ovulation in any teleost species. In this sense, my current study deals for the first time with the expression of ovulation-related genes in teleosts.

In Chapter 1, I showed that *EP4b* mRNA was induced by an LH/cAMP-mediated pathway. DHP, which is the physiological progestin hormone in medaka, was also required to induce expression of *EP4b*. An interesting finding of my study is that, like *EP4b* mRNA, nPR was induced by the LH/cAMP-mediated pathway and the expression of nPR preceded that of *EP4b*. Based on these findings, I hypothesized that nPR could be an important regulator of *EP4b* expression. Indeed, a ChIP assay elaborated for the whole follicle tissues of the fish demonstrated direct binding of nPR to the promoter region of EP4b gene approximately 12 h before ovulation. This timing corresponds to the point at which intrafollicular DHP levels are expected to reach their maximum. Therefore, I conclude that *EP4b* mRNA expression is under the control of nPR.

In Chapter 2, I investigated the possible involvement of membrane progestin receptors (mPRs) in the expression of *EP4b* mRNA in the follicle. *mPRα* and *mPRγ* were constitutively expressed in the preovulatory follicle GCs. I found that mPRα decreases intercellular cAMP levels in response to DHP more effectively than does mPRγ through *in vitro* experiments using HEK293T cells. Furthermore, the treatment of the follicles with 1 μM FSK under *in vitro* conditions inhibited both *EP4b* mRNA induction and ovulation. Because oocyte maturation was not affected by 1 μM FSK, FSK's effect on the expression of *EP4b* mRNA was considered to be selective. Therefore, these data strongly suggested an involvement of mPRα in *EP4b* expression in GCs. Taken together, I concluded that not only nPR but also mPR (most likely mPRα) are involved in the LH-induced expression of *EP4b* in the follicles.

P4 plays important roles in ovarian function of a wide variety of vertebrate animals [Furr et al., 1978]. In mammalian species, 17β-estradiol, which is produced in the GCs [Moon et al., 1985; Spicer et al., 2002], is essential for folliculogenesis [Lubahn et al., 1993]. LH surges induce a dramatic shift in the concentrations of sex steroids. The follicle cells of preovulatory follicles begin to produce a significant amount of P4 within a few hours after the ovulatory process has been initiated by an LH surge [Bahr, 1978]. This shift results from changes in the levels of expression and/or activation of enzymes, such as the steroid 17-α-monooxygenase (Cyp17a1), p450 side-chain cleavage enzyme (p450-scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD), which are involved in the steroidogenesis in response to LH [Saidapur and Greenwald, 1979; Chouhan et al., 2014]. P4 is reported to be involved in not only reproductive events but also anti-apoptotic activity [Peluso and Pappalardo, 1998; Makrigiannakis et al., 2000]. Similarly, a dynamic shift in steroidogenesis from 17β-estradiol to DHP is observed in

medaka [Senthilkumaran et al., 2004; Fukada et al., 1994]. 17β-estradiol stimulates the biosynthesis of vitellogenin, a yolk protein precursor vital for follicle growth [Miyamoto et al., 2003]. This shift is triggered by LH, which induces the expression of 20β- hydroxysteroid dehydrogenase probably through the activation of cAMP response element binding protein (CREB), and, as a result, the production of 17β-estradiol catalyzed by P450 aromatase (P450 arom) is reduced. As an additional effect of DHP in teleost ovaries, the reduction of apoptosis of GCs and TCs by DHP was recently reported in Atlantic croaker [Dressing et al., 2010].

Both *mPRα* and *mPRγ* are constitutively expressed in the preovulatory follicles [Chapter 2]. Consistent with this observation, no significant changes in the expression levels of these *mPRs* were observed when the preovulatory follicles, which had been isolated before *in vivo* LH stimulation, were treated *in vitro* with recombinant LH. These results indicate that, unlike nPR, the expression of mPRs in the fish preovulatory follicles is LH-independent.

Because of the constitutive expression of mPRs in the follicles, it is conceivable that DHP treatment alone may induce oocyte maturation of follicles without *in vivo* LH stimulation, and I confirmed that this was indeed the case (my unpublished data). Taking into account the fact that mPRs are constitutively expressed in the preovulatory follicles, mPRs may have a role in other biological process(es), in addition to its well-known role in oocyte maturation. As recently reported for the Atlantic croaker [Dressing et al., 2010], mPRs may also protect medaka oocytes or follicle cells from apoptosis. Such anti-apoptotic roles for mPRs might be protective for the oocytes and follicle cells of growing follicles because *mPR* transcripts are detected in the follicles at the vitellogenic stage (my unpublished data).

Previous studies using mammalian species have investigated the relationship among nPR, PGE_2 and LH. In the mouse ovary, nPR is induced by LH in the preovulatory follicle GCs [Rose et al., 1999]. LH surges also induce COX-2 in the GCs and cumulus cells of the follicles, together with increases in follicular PGE_2 levels [Sirois, 1994]. Though nPR and COX-2 are simultaneously induced by gonadotropin, there is no report indicating that nPR regulates the expression of COX-2. In the medaka ovary, no evidence for a close relationship in the expression of these molecules has thus far been demonstrated. Rather, *COX-2* was found to be constitutively expressed in fish follicles in contrast to mammalian COX-2. As demonstrated in this study, the effect of PGE_2 on fish ovulation is regulated through the synthesis of its receptor EP4b. Interestingly, the expression of *EP4b* is under the control of nPR as demonstrated by a ChIP assay. At present, it is not known whether this mechanism by which PGE_2 effects on the follicles are regulated through nPR-mediated PGE_2 receptor expression is unique to medaka. To answer this question, further studies using other teleost species are required. There is a clear morphological difference between mammalian and teleost ovarian follicles. One may speculate that such differences in follicular tissue structures might result in different regulatory mechanisms, which could be resolved by further studies. Nevertheless, an important role for PGE_2 in successful ovulation is conserved among vertebrate animals including mouse and medaka.

It is proposed that a sequential two-step ECM protein hydrolysis mechanism could explain the process of follicle rupture that occurs during ovulation in medaka. In the first step, which occurs approximately 7 h before ovulation, the protease plasmin is generated by urokinase-type plasminogen activator (uPA) and the activated plasmin enzyme decomposes laminin, which is a major component of the basement membrane

in the follicle layer. Laminin hydrolysis only lasts for a few hours. Plasmin activity is then terminated by the production of plasminogen activator inhibitor-1(Pai-1), which is induced by LH stimulation of GCs approximately 5 h before ovulation [Ogiwara et al., 2012; Ogiwara et al 2014]. MMPs are then induced or activated and hydrolyze type Ⅳ and type Ⅰ collagen present in ECM of the follicle layer [Ogiwara et al., 2005].

While ECM hydrolysis takes place in the apical region of ovulating follicles, PGE_2-mediated reactions proceed independent of ECM hydrolysis. At present, the mechanism behind PGE_2-involving processes that facilitate ovulation in the follicles is not defined. However, several pieces of evidence indicate that PGE_2 might be involved in the reorganization of actin fibers in the follicle cells at the time of ovulation (unpublished results from my laboratory).

It is generally understood that in teleost fish ovaries, mPRs mediate non-classical progestin activity to induce oocyte maturation [Thomas et al., 2002], while nPR activates a classical genomic mechanism involving steroids. The latter mechanism induces the synthesis of various proteins in association with follicle cell ovulation. Indeed, MT2-MMP [Ogiwara et al., 2013] and Pai-1 [Ogiwara et al., 2014], both of which are shown to be indispensable for medaka follicle ovulation, are induced in the ovulating follicle GCs by LH stimulation. We found that nPR acted as an intermediary during the synthesis of these proteins (unpublished results from my laboratory). My study showed that EP4b was another example of a protein synthesized in an LH-induced and nPR-mediated manner in the follicle cells. An interesting finding of my study was that *EP4b* mRNA expression in GCs was strongly inhibited by 1 μM FSK, which was without effect on oocyte maturation [Chapter 2], suggesting a possible involvement of mPRs in *EP4b* mRNA expression. Under the same concentrations, *Pai-1* mRNA

expression was accelerated by approximately 1-3 h while *MT2-MMP* mRNA expression was not affected (my unpublished data). These findings indicate that mPRs possibly contribute to the expression of at least *EP4b* and *Pai-1* mRNA in the follicle GCs. In any case, my results indicate that both mPRs and nPR are necessary for the expression of some genes in the preovulatory follicle GCs after LH stimulation.

This is the first study reporting the role of nPR, and perhaps mPRs too, in *de novo* mRNA synthesis preovulatory follicle GCs using the teleost medaka. The results of my study have solved an important problem in determining how the expression of EP4b, an ovulation-related gene/protein, is regulated in the follicle cells of preovulatory follicles. The precise mechanism of the involvement of both nPR and mPRs in EP4b mRNA expression in the follicle cells remaining to be defined.

I believe that my present study definitely contributes to the understanding of ovulatory mechanisms of the teleost medaka.

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