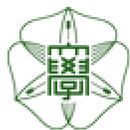
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Expression of Membrane Progestin Receptors (mPRs) in Granulosa Cells of Medaka Preovulatory Follicles

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Membrane progestin receptor (mPR) α on the cell membrane of the oocyte is involved in the meiotic maturation of vertebrates, including teleosts, but little is known about the role of this membrane-bound follicular receptor. We investigated the ovarian expression of membrane progestin receptor (mPR) mRNA in medaka. In follicles that were destined to ovulate, transcripts of *mPRα* and *mPRγ* were expressed in the oocytes as well as the granulosa cells. Transcripts of *mPRα* and *mPRγ* were expressed at relatively constant levels in the whole ovary and in the preovulatory follicles throughout the 24-h spawning cycle. In vitro incubation of the preovulatory follicles with recombinant medaka luteinizing hormone caused no significant changes in the expression of *mPRα* and *mPRγ* mRNA, suggesting LH-independent follicular expression of these mPR genes. Using HEK293T cells expressing medaka mPRs, forskolin-elevated intracellular cAMP levels were found to be reduced on treatment of the cells with ligand 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), but only in the cells expressing mPRα. These results indicate that activation of mPRα and mPRγ with DHP may cause differential effects on the granulosa cells. Information obtained from the present study may help to elucidate the role of mPRα and mPRγ in the granulosa cells of the follicles.

Key words: medaka, ovulation, mPRα, PGE_2 receptor, expression

INTRODUCTION

Progestins are essential for reproduction in both male and female vertebrates. In mammals, steroids trigger a series of events that lead to ovulation (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., 2003b; Thomas et al., 2004; Tokumoto et al., 2006; Nagahama and Yamashita, 2008). Additionally, 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 have revealed that these effects are mediated either by the classical nuclear progestin receptor (nPR) or by membrane-bound progestin receptors (mPRs) (Nagahama and Yamashita, 2008; Thomas, 2012; Wang et al., 2014).

We previously reported that prostaglandin E_2 signaling is required for ovulation in the teleost medaka (Fujimori et al., 2011; Fujimori et al., 2012). Our studies also demonstrated that the expression of the prostaglandin E_2 receptor subtype EP4b, but not its ligand-producing enzyme COX-2, is dramatically induced in the preovulatory follicles as ovulation time approaches, suggesting a mechanism by which the effect of prostaglandin on medaka ovulation is regulated by *de novo* synthesis of the EP4b receptor protein. Our previous finding that COX-2 activities and PGE_2 levels are fairly constant in the granulosa cells of preovulatory follicles that are destined to ovulate (Fujimori et al., 2012) is consistent with this idea. More recently, medaka ovulation was shown to be triggered by exposing the preovulatory follicles to luteinizing hormone (LH) approximately 17 h before ovulation during the 24-h spawning cycle (Ogiwara et al., 2013), and it was also demonstrated that the synthesis of the transcription factor nPR precedes the induction of *EP4b* mRNA in the granulosa cells of preovulatory follicles that have undergone the LH surge (Hagiwara et al., 2014). Additionally, we found that the expression of *EP4b* mRNA is under the control of nPR, which is activated by binding to the ligand 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), a naturally occurring maturation-inducing hormone (MIH) in medaka (Sakai et al., 1988).

In our previous attempts to localize *nPR* mRNA in the medaka ovary (Hagiwara et al., 2014), we observed the expected follicular expression of *mPRα* (i.e., Paqr7). Further examinations revealed that *mPRα* (*paqr7*) and *mPRγ* mRNA (*paqr5*) are expressed at substantial levels in the follicle cells of ovulatory follicles. We therefore felt it reasonable to assume that the nuclear and membrane-bound progestin receptors associated with follicle cells would be simultaneously activated when intrafollicular DHP levels are elevated following the LH surge. mPRs activated under these conditions may have a role in biological processes associated with the follicle cells of LH-stimulated preovulatory follicles. No previous studies reported to date have focused on the roles of mPRs in ovulation, which contrasts with the inten-

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sive studies of mPRα in terms of its involvement in oocyte maturation (Zhu et al., 2003a; Zhu et al., 2003b; Thomas et al., 2004; Tokumoto et al., 2006; Nagahama and Yamashita, 2008). In the present study, as a first step to approach this problem, we investigated in detail the expression and localization of the membrane-bound progestin receptors in the medaka. These results, together with the results of partial in vitro characterization studies of fish mPRs are reported in this paper.

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 cycle; temperature, 27°C). The ovaries and other tissues were removed at the indicated time points after a 24-h spawning cycle was established. The isolation of the preovulatory follicles and preparations of the 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 study 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, in the post-vitellogenic phase, stage IX) were isolated and pooled from the ovaries of three to five fish 22 h before ovulation. Approximately 20 follicles were used per culture. Follicles were cultured at 26–27°C 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 recombinant luteinizing hormone (rLH) as previously described (Hagiwara et al., 2014).

Preparation of the granulosa cells from the spawning medaka ovaries

Preovulatory follicles were collected from the ovaries of spawning female medaka 3 h before ovulation as previously described (Ogiwara et al., 2013). In brief, the follicle layers consisting of both granulosa cells and theca cells were separated from the oocytes using forceps. After washing three times with PBS, the tissues were placed in phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.25% trypsin followed by gentle rotation for 30 min at room temperature. Treated samples were collected by centrifugation at 2000 rpm for 3 min. After three washes with 90% medium 199 solution, the precipitates containing the granulosa and theca cells were suspended in the same medium and filtered with 100-μm nylon filters (BD Bioscience, Bedford, MA). The resultant filtrates were cultured in medium containing 50 μM gentamycin and 5% carp serum. After culturing for 48 h, unattached cells were removed by gentle washing with PBS, and the cells that remained attached to the dish were collected. The cells obtained in this manner were granulosa cells derived from follicles that were predicted to ovulate (Kato et al., 2010). Total RNA for RT-PCR analysis was isolated from the collected cells.

RT-PCR and real-time RT-PCR

Total RNAs were separately prepared from the various tissues of the 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 complementary DNA (cDNA) prepared as above was used for PCR and real-time PCR. The cDNAs were PCR amplified using the TaKaRa Ex Taq® Hot Start Version (Takara, Tokyo, Japan) as previously described (Hagiwara et al., 2014). Real-time RT-PCR experiments were conducted using an ABI 7300 Real-Time PCR System (Life Technologies, Inc., Rockville, MD). The amplification efficiencies were verified using the same system. The PCR reactions and data analyses were performed according to previously described procedures (Hagiwara et al., 2014). The gene transcript levels in the tissues were normalized to those of the housekeeping genes *cytoplasmic actin (actb)* or *ribosomal protein L7* (*rpl7*). The primers used for RT-PCR and real-time RT-PCR are listed in Table 1.

Table 1. Primer nucleotide sequences used.

Gene	Primer sequences Forward (F) and Reverse (R) primers (5′-3′)	Accession No.
For Real-time RT-PCR		
mPRα	F: CCACGTTCTCTACGGCTACA	NM_001177476
mPRα	R: CCAATCTGCTCCATCACAAC	
mPRβ	F: GGAAGAGAGTCGGTCTGAGGTT	NM_001201495
mPRβ	R: CAAGGACGAGGTCTGAGATGTG	
mPRγ	F: CCGTCAAAAGATCAATCTGT	NM_001201493
mPRγ	R: CGCCCAATGCTCTCCATCAC	
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	
α1(I)	F: GAGAAATCTGGACTTGAA	AB 280535
α1(I)	R: GTACAGAGCAACCGAGTT	
actb	F: CAGACACGTATTTGCCTCTG	D89627
actb	R: CAAGTCGGAACACATGTGCA	
For *in situ*hybridization		
mPRα	F: TGCAAGTGCGTGAACCGCAGC	NM_001177476
mPRα	R: ATGTGACGTGATCGATGACTA	
mPRγ	F: CGGCTCCTACCTGGATTACGT	NM_001201493
mPRγ	R: CTTGTATGTTCAGCAGGCCGA	

α1(*I*), collagen type I α1-chain.

In situ hybridization

The RNA probes were prepared by in vitro transcription of the reverse transcriptase fragments of *mPRα* and mPRγ with T3 or T7 RNA polymerase using a digoxigenin (DIG) RNA labeling mix (Roche Diagnostics, Basel, Switzerland). The RT-PCR primers used for probe preparation are listed in Table 1. Ovary cryostat sections (12 μm) from medaka were thaw-mounted onto slides coated with silane. The sections were fixed in 4% paraformaldehyde (Wako, Osaka, Japan) in PBS for 15 min at room temperature and washed with PBS three times. The sections were acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine/HCl buffer (pH 8.0). Hybridization was conducted at 60°C for 18 h in 50% formamide, 0.5 M NaCl, 10 mM TRIS-HCl (pH 8.0), 10% dextran sulfate, 1× Denhardt's solution, 0.25% sodium dodecyl sulfate, and 0.2 mg/ml yeast transfer RNA. The sections were consecutively washed at 60°C in 50% formamide/2× standard sodium citrate (SSC) for 30 min, at 60°C in 2× SSC for 20 min, and at 60°C in 0.2× SSC for 20 min. The hybridization probes were detected by using a Dig Nucleic Acid Detection Kit (Roche).

GloSensor cAMP assay of mPRs

The coding regions of *mPRα* and *mPRγ* were amplified by PCR of 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 resulting vectors, i.e., pCMV-mPRα or pCMV-mPRγ, were confirmed by sequencing.

HEK293T cells (RIKEN cell bank, Tukuba, Japan) were cultured at 37°C in DMEM (Wako) supplemented with 10% FBS (Wako), 1× penicillin-streptomycin-amphotericin B suspension (Wako), and 2 mM L-glutamine (Wako). The cells were transfected with Glosensor™-22F cAMP plasmid (Promega, Madison, WI) expressing a modified form of luciferase, which contained 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. Forty-eight hours after transfection, the medium was removed and replaced with fresh medium containing 1% Glosensor™ 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 activities of the cells were 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 min.

Statistical analyses

All experiments were conducted at least three times to confirm the reproducibility of the results. Statistical significance was determined using Student's *t*-tests or one-way ANOVA, as appropriate. Post hoc testing was performed with Tukey's t-test using Microsoft Excel. The data are presented as the mean ± the SEM. $P < 0.05$ was considered to be statistically significant.

RESULTS

mRNA expression levels of mPRs in the various medaka tissues

Three mPR genes (*mPRα*, *mPRβ* and *mPRγ*) were identified in medaka using a search tool from the National Center for Biotechnology Information (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*. These observations were further substantiated by real-time RT-PCR analysis (Fig. 1A). The *mPRγ* transcripts were the most abundant of the genes that were analyzed in the fish ovary. *mPRα* mRNA was also detected, but its abundance was approximately one-third that of *mPRγ*. The expression of *mPRβ* mRNA was very low compared to *mPRα* and *mPRγ*. Based on these findings, subsequent studies investigated only mPRα and mPRγ.

mPRα and *mPRγ* mRNAs were both expressed in fish ovaries and testes (Fig. 1B and 1C). The tissue distribution of medaka *mPRα* mRNA was very similar to that reported for the spotted seatrout in which *mPRα* was exclusively expressed in the reproductive and neuroendocrine tissues (Zhu et al., 2003b; Nagahama and Yamashita, 2008). Wider tissue distributions of *mPRα* mRNA expression have been reported for other organisms, such as the goldfish (Tokumoto et al., 2006). These results suggest that mPRα and mPRγ play roles in the medaka reproductive organs and brain.

Expression of mPRα and mPRγ transcripts in fish ovary

The changes in the expression levels of *mPRα* and *mPRγ* were examined using whole fish ovaries with established 24 h-spawning cycles by real-time RT-PCR. The *mPRα* and *mPRγ* mRNAs were expressed at relatively con-

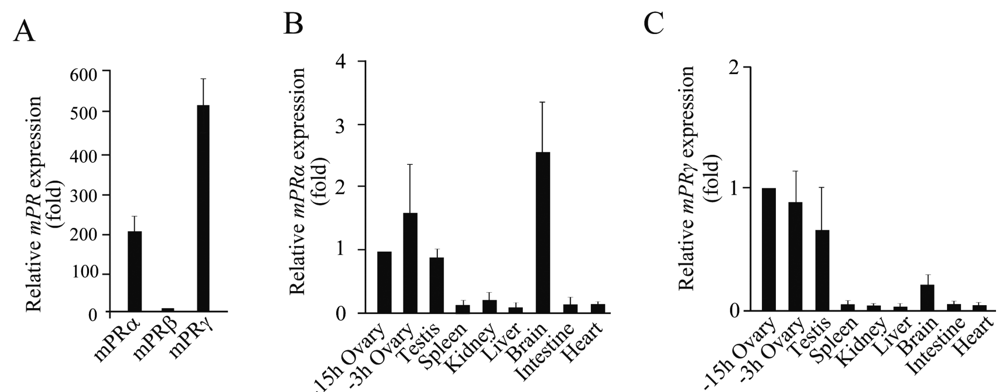

Fig. 1. mRNA expression levels of *mPRs* in various medaka tissues. **(A)** Expression levels of *mPRα*, *mPRβ* and *mPRγ* transcripts were determined by real-time RT-PCR using total RNA isolated from the ovaries of spawning female medaka 15 h before ovulation. The expression levels were normalized to those of *actb* and are presented as the fold-changes relative to the *mPRβ* transcript levels. The results are presented as the means ± the SEM ($n = 5$). **(B)** Expression levels of the *mPRα* transcripts were determined by real-time RT-PCR using total RNA isolated from various tissues at an 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 are presented as the fold-changes relative to the level of each gene in the –15 h ovary. Results are presented as the mean ± the SEM ($n = 5$). **(C)** Expression levels of *mPRγ* transcripts were determined as in **(B)**. The results are presented as the mean ± the SEM ($n = 5$).

stant levels throughout the spawning cycle (Fig. 2A and 2B). Transcripts of the two membrane progestin receptors were also analyzed using post-vitellogenic follicles that were destined to ovulate (Fig. 3A and 3B). *mPRα* transcript levels did not change during the spawning cycle (Fig. 3A). *mPRγ* expression was low during the early post-vitellogenic stage (–23 h) but appeared to be more abundant thereafter (–19 h to –3 h, Fig. 3B). The difference was not statistically significant under the conditions because of the large standard errors due to the individual differences. The expression of *mPRγ* might show an increasing pattern toward ovulation when experiments are added. Nevertheless, these results indicate that both the *mPRα* and *mPRγ* transcripts were expressed at detectable levels in the fish preovulatory follicles.

The in situ localization of the progestin receptor mRNA was examined using frozen sections of spawning fish ovaries that were isolated 3 h after ovulation. Staining with antisense and sense probes for *mPRα* was performed using neighboring sections in the same conditions. The antisense probe detected strong signals that were associated with the oocyte cytoplasm of small- and medium-sized growing follicles (Fig. 4A, left). In the large preovulatory follicles, the oocyte cytoplasm, which was present as a very thin layer between the egg membrane and the yolk that occupied a large central space of the egg, exhibited weak but detectable staining. This observation is consistent with previous reports of other teleost species in which the oocytes of preovulatory follicles express *mPRα* (Thomas, 2012). Positive signals were also observed in the follicle layers of the large preovulatory follicles. Follicle layers of follicles with diame-

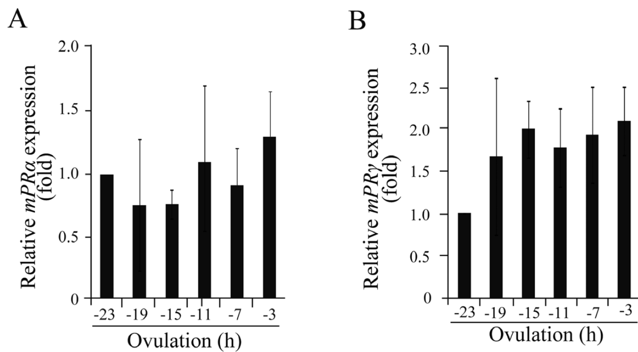

Fig. 2. The expression of *mPRα* and *mPRγ* mRNAs in the ovary. **(A)** Real-time RT-PCR analyses of *mPRα* were performed using total RNA isolated at the indicated time points from the ovaries of adult medaka on a 24-h spawning cycle. The expression levels of *mPRα* were normalized to those of *actb* and are expressed as the fold changes compared to the levels of the –23 h ovary. The results are presented as the mean ± the SEM (n = 5). The *mPRα* transcript levels of ovaries at –19, –15, –11, –7 and –3 h are not significantly different from those of the –23 h ovary. **(B)** Real-time RT-PCR analysis of *mPRγ* was performed as detailed in **(A)**. The results are presented as the mean ± the SEM (n = 5). The *mPRγ* transcript levels of ovaries at –19, –15, –11, –7 and –3 h are not significantly different from those of the –23 h ovary.

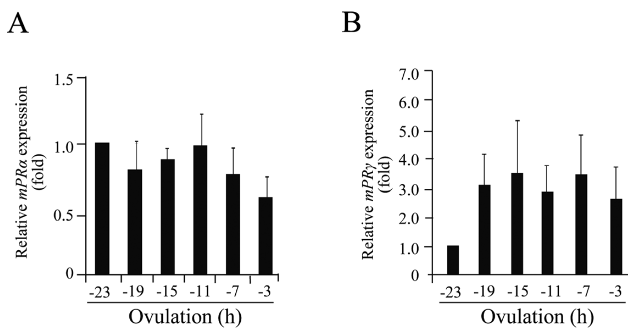

Fig. 3. The expression of *mPRα* and *mPRγ* mRNAs in the ovulatory follicle. **(A)** Real-time RT-PCR analyses of *mPRα* were performed using total RNA isolated from the preovulatory follicles of adult medaka ovaries at various times before ovulation. The expression levels of *mPRα* were normalized to those of *actb* and are expressed as the fold changes compared to the levels of the –23 h follicles. The results are presented as the mean ± the SEM (n = 5). The *mPRα* transcript levels of preovulatory follicles at –19, –15, –11, –7 and –3 h are not significantly different from those of the –23 h follicles. (B) Real-time RT-PCR analyses of *mPRγ* were performed for as detailed in (A). The results are presented as the mean ± the SEM (n = 4). The *mPRγ* transcript levels of preovulatory follicles at –19, –15, –11, –7 and –3 h are not significantly different from those of the –23 h follicles.

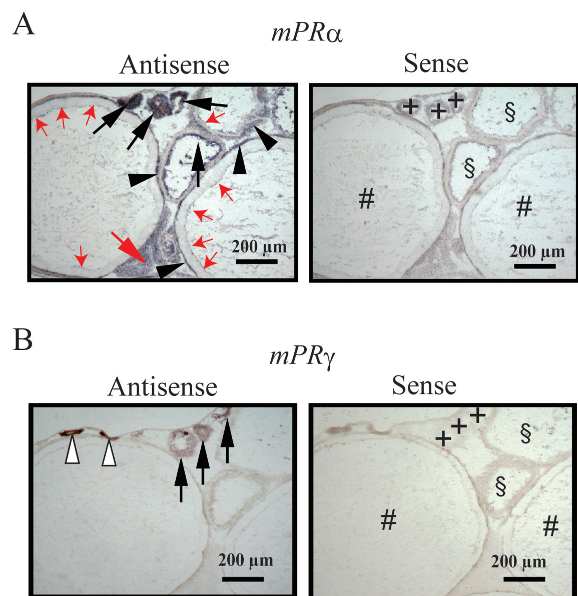

Fig. 4. In situ detection of *mPRα* and *mPRγ* mRNAs in the medaka ovary. **(A)** In situ hybridization analyses for *mPRα* were performed using frozen sections of ovaries that were isolated 12 h before ovulation. Antisense (left) and sense probes (right) were used to detect the signals. Clear positive staining is associated with the oocyte cytoplasm (large arrows) of small-sized (+), the middle-sized growing follicles (§), the follicle layers (arrowheads) of the middle-sized and large preovulatory follicles (#) and the follicle tissues of the postovulatory follicles that had just ovulated (large red arrow). Weak positive signals were also detected in the oocyte cytoplasm (small red arrows) of the middle-sized and large preovulatory follicles. A representative result of three independent experiments is shown. **(B)** In situ hybridization analyses were performed for *mPRγ* as in **(A)**. Positive staining is associated with the oocyte cytoplasm (large arrows) of the small-sized growing follicles (+). The capillaries (white arrowheads) in the follicle wall exhibit positive staining. Middle-sized (§) and large-sized follicles (#) are indicated. A representative result of three independent experiments is shown.

ters greater than 300 μm also exhibited clear signals. Additionally, signals were clearly observed in the postovulatory follicles that had just released their oocytes. The *in situ* detection of *mPRγ* transcripts with an antisense probe revealed signals in the oocyte cytoplasm of the small-sized follicles and capillaries in the follicle wall. Under these conditions, neither the mid-sized nor the large follicles exhibited detectable staining, suggesting that the smaller follicles expressed *mPRγ* mRNA in an overwhelmingly large abundance relative to the mid-sized and large follicles.

To gain further insight into the expression of the *mPRα* and *mPRγ* mRNAs in the preovulatory follicles, we compared their expression levels in the ovary, the follicle, the follicle layer and the follicle layer-derived granulosa cells. Total RNA was prepared and used for real-time RT-PCR analyses of *mPRα* and *mPRγ* mRNA expression. The amplification efficiencies of the real-time RT-PCR for the *mPRα* and *mPRγ* primer sets were comparable. Analysis was conducted using total RNA prepared from intact –22 h ovaries and ovaries from which large preovulatory follicles had been physically removed by a pair of tweezers. The expression ratios of *mPRγ/mPRα* was approximately 2.5 and 0.25 in intact ovaries and ovaries without large follicles, respectively (data not shown), suggesting that large preovulatory follicles strongly express *mPRγ* mRNA. Consistent with the above results, the *mPRγ* transcript levels were greater than those of *mPRα*, not only in the whole preovulatory follicle but also in the follicle layer (Fig. 5A and 5B). The expression of *mPRα* and *mPRγ* in the granulosa cell fractions isolated from the follicle layer with the culture method was determined by RT-PCR (Fig. 5C). The transcript levels of *MT3-MMP* (an oocyte marker), *gelatinase B* (a marker of granulosa cells), c*ollagen type I α-chain* (a marker of theca cells) and *actb* (a control housekeeping gene) were used as controls (Fig. 5C). Among the assessed marker genes, only *gelatinase B* transcripts were detected, indicating that the granulosa cells were isolated reliably. The *mPRα* and *mPRγ* transcripts were successfully amplified from the cell fraction. Further real-time RT-PCR analysis using the same fraction showed a higher expression of *mPRγ* than *mPRα* (Fig. 5D). These results indicated that both of the progestin receptor mRNAs were expressed in the granulosa cells of the preovulatory follicles.

Collectively, the above results suggest that mPRα and mPRγ may be involved in granulosa cell function in the preovulatory follicles that are predicted to ovulate.

Effects of rLH on the expression of mPR mRNA in the preovulatory follicle

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, the levels of the *mPRα* transcripts in the rLH-treated follicles were slightly greater than those in the rLH-untreated follicles. The average values at each time point were 2–6 times greater in the rLH-treated follicles than in the untreated follicles, but these changes were not significant (Fig. 6A). Similar in vitro experiments were conducted to determine the follicular expression of *mPRγ* mRNA (Fig. 6B). rLH had no effect on the follicular expression of *mPRγ*, indicating that *mPRα* and *mPRγ* mRNA expression may not be under the control of LH.

In vitro characterization of medaka mPRα and mPRγ using HEK293T cells

To examine the initial events triggered by the activation of medaka mPRs, HEK293T cells that transiently expressed medaka mPRα or mPRγ were prepared. FSK treatment of the cells expressing mPRα increased intracellular cAMP levels, and the cyclic nucleotide levels peaked within 3 min. The FSK-elevated nucleotide levels gradually decreased (Fig. 7A). However, intracellular cAMP levels decreased immediately when DHP was added to the reaction.

In the cells that expressed medaka mPRγ, DHP treatment did not affect FSK-elevated intracellular cAMP levels (Fig. 7B). As a control, experiments with HEK293T cells that had not previously been transfected with any progestin receptor cDNA were conducted. The addition of DHP to the

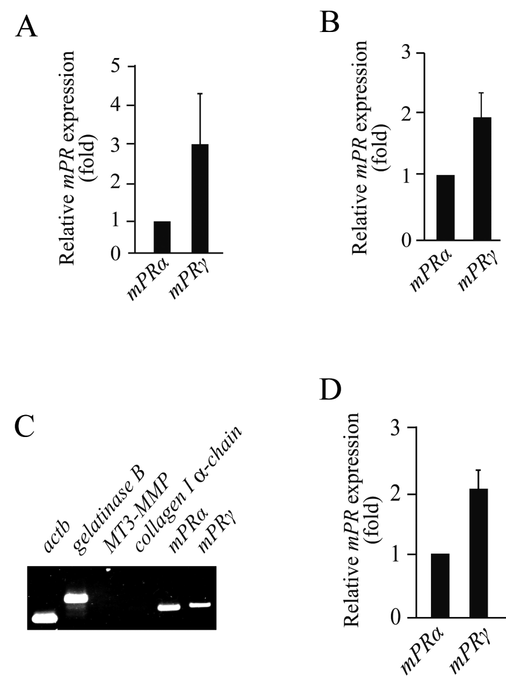

Fig. 5. Expressions of *mPRα* and *mPRγ* mRNAs in the preovulatory follicle GCs. **(A)** Real-time RT-PCR analyses were performed for *mPRα* and *mPRγ* using total RNA isolated 7 h before ovulation from the preovulatory follicles of adult medaka on 24-h spawning cycle. The expression levels were normalized to the levels of the *actb* transcript, and the relative expression levels are expressed. The results are the mean ± the SEM (n = 5). (B) Real-time RT-PCR analyses were performed for *mPRα* and *mPRγ* using total RNA isolated from the follicle layers of the preovulatory follicles 7 h before ovulation. The normalization and presentation of the results are as in (A). The results are presented as the mean ± the SEM (n = 5). (C) RT-PCR analyses were performed using total RNA isolated from the granulosa cells of the preovulatory follicles following the application of our culture method. The transcripts of *mPRα* and m*PR*γ were detected. The transcripts of *MT3-MMP* (a marker of oocytes), *gelatinase B* (a marker of granulosa cells), *collagen type I α-chain* (a marker of theca cells) and *actb* (a control house-keeping gene) were also determined for comparison. A representative result of three independent experiments is shown. (D) Real-time RT-PCR analyses were performed for *mPRα* and *mPRγ* using the total RNA prepared from the granulosa cells as in (C). The normalization and presentation of the results are as in (A). The results are presented as mean ± SEM (*n* = 4).

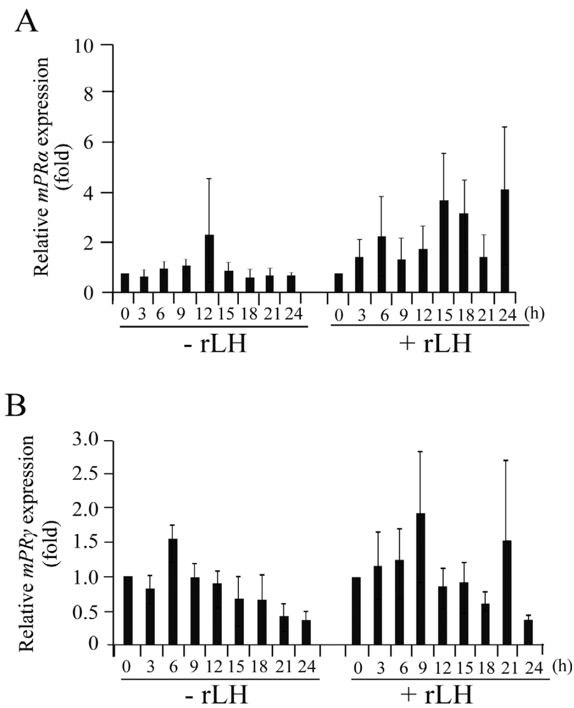

Fig. 6. The effects of rLH on the expression of *mPR* mRNA in the large follicle. **(A)** Preovulatory follicles were isolated from spawning medaka ovaries 22 h before ovulation and incubated with medaka rLH (100 µg/ml). The total RNA was isolated from the in vitro incubated follicles that were obtained at the indicated time points and used for real-time RT-PCR analyses of *mPRα* mRNA. The expression levels were normalized to those of *actb* and are expressed as the fold changes relative to the expression levels of the –22 h follicles that correspond to the 0 h follicles. The results are presented as the mean ± the SEM (n = 4). The *mPRα* transcript levels of preovulatory follicles that had been incubated with rLH for 3–24 h are not significantly different from those of the 0 h follicles. **(B)** Real-time RT-PCR analyses were performed for *mPRγ* expression. The treatment of the follicles with rLH, total RNA isolation and normalization and presentation of the data are, as in **(A)**. The results are presented as the mean ± the SEM (n = 4). *mPRγ* transcript levels of preovulatory follicles that had been incubated with rLH for 3–24 h are not significantly different from those of the 0 h follicles.

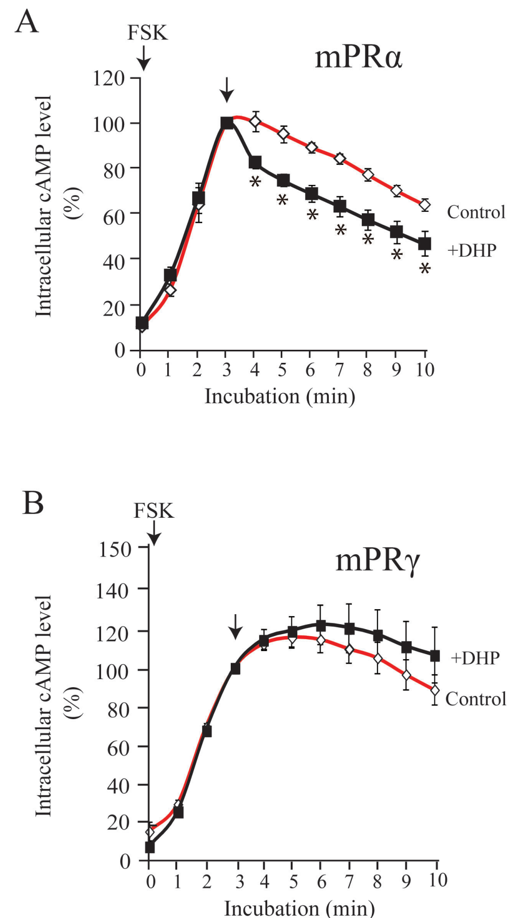

Fig. 7. In vitro characterization of medaka mPRα and mPRγ using HEK293T cells. **(A)** HEK293T cells transfected with Glosensor™-22F cAMP plasmid and pCMV-mPRα were treated with FSK (1 µM) at room temperature in PBS. After 3 min of incubation with FSK, the cells were further incubated in the presence or absence of DHP (1 µM) for 7 min. The luciferase activities of the cells, which represents the 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%. The relative levels of cAMP are shown. The results are presented as the mean ± the SEM (n = 4). The asterisks denote significant differences at $P < 0.05$. **(B)** HEK293T cells transfected with Glosensor™-22F cAMP plasmid and pCMV-mPRγ were used. The treatment of the cells with FSK and subsequently with DHP were as described in **(A)**. The luciferase activities of the cells were monitored, and the results are presented as the mean ± the SEM (n = 4).

cells that exhibited elevated intracellular cAMP levels did not result in an immediate decrease in nucleotide levels (data not shown), suggesting that the progestin receptors that were intrinsic to the HEK293T cells, if any were present, had no effect on the results of our experiments. The above results imply that the binding of DHP to medaka mPRα but not mPRγ caused decreases in intracellular cAMP levels.

DISCUSSION

Since mPRα was first identified as a novel membrane-bound progestin receptor involved in meiotic maturation in spotted seatrout (Zhu et al., 2003b), its existence has been demonstrated in a variety of species, including mammals (Zhu et al., 2003a; Tokumoto et al., 2006; Thomas et al., 2007; Dressing et al., 2012). However, there have been no reports of medaka mPRα expression to date. In the present study, we confirmed that the oocytes of the preovulatory follicles of the spawning medaka expressed *mPRα*. Additionally, we could show that *mPRα* and *mPRγ* are expressed in the granulosa cells of the follicles.

The *mPRα* mRNA levels were fairly constant in the fish follicles throughout the 24-h spawning cycle while the *mPRγ* mRNA levels increased as ovulation approached. However, the follicular levels of the *mPRα* and *mPRγ* transcripts were not affected by rLH treatment in vitro, suggesting that *mPRα* and *mPRγ* expression is regulated in an LH-independent manner in the follicles. We suggest that both membrane progestin receptors are expressed, at least in the follicles that enter the post-vitellogenic stage of medaka. Based on this idea, we believe the cellular effects that are exerted through the mPRs are primarily dependent on the availabilities of

their specific ligands. However, as documented in previous studies by other investigators, ovarian follicles from the spotted seatrout (Zhu et al., 2003b), Atlantic croaker (Tubbs et al., 2010) and goldfish (Tokumoto et al., 2006) exhibit increased mPR mRNA and protein expression following gonadotropin treatment.

In situ hybridization analysis indicated that clear positive signals for *mPRα*, but not for *mPRγ*, were detected in the follicle layers of the large preovulatory follicles while real-time RT-PCR analysis showed that *mPRγ* transcript levels were greater than those of *mPRα* in the layer. In this context, we observed that the follicle layers of the follicles expressed two different species of *mPRγ* mRNA by PCR amplification (our unpublished observation): a 1.4 kb transcript and a 1.0 kb transcript. The latter transcript lacks approximately 400 bp nucleotides in the 3' region compared with the former. Because our probe used for in situ hybridization analysis of *mPRγ* mRNA was designed and synthesized based on the sequence of the 3' noncoding region of the gene, it might have hybridized only with the 1.4 kb *mPRγ* mRNA. This may explain our failure to detect a clear *mPRγ* signal associated with the follicle layers of the preovulatory follicles.

Oocyte maturation in fish is initiated in vivo by the exposure of preovulatory follicles to LH, resulting in the induction of the synthesis of DHP, which is known as MIS in most teleost species (Nagahama and Yamashita, 2008). The binding of DHP to mPR on the cell membrane of the oocyte activates the Giα protein, which in turn reduces intracellular cAMP levels in the oocyte and eventually leads to the formation and activation of maturation-promoting factor (MPF) (Zhu et al., 2003a; Zhu et al., 2003b; Nagahama and Yamashita, 2008). The MPF then triggers subsequent maturation processes that consist of GVBD, chromosome condensation, the assembly of the meiotic spindle and the formation of the first polar body. Of the two medaka mPRs, it is likely that only mPRα acts as a signal transducer by converting the DHP-binding event into intracellular signals in the oocyte, as the present study demonstrated that mPRα, but not mPRγ, caused a reduction in intracellular cAMP levels. Consistent with this observation, the expression of *mPRα* mRNA in the oocyte cytoplasm of the large preovulatory follicles was confirmed by in situ hybridization analysis. At present, evidence for the presence of mPRα proteins that are associated with the plasma membrane of the oocyte is still lacking and remains for future investigation.

In medaka, a drastic rise in DHP levels in the preovulatory follicle occurs approximately 5 h after the LH surge (Sakai et al., 1988; Ogiwara et al., 2013). The expression of the transcription factor nPR is also induced by the LH surge in the follicular granulosa cells by the time the steroid hormone levels peak (Hagiwara et al., 2014). nPR, which is activated by associating with DHP, plays essential roles in the activation of various genes that are necessary for ovulation. Because *mPRα* and *mPRγ* transcripts and, presumably, their corresponding proteins are expressed in the granulosa cells of the follicles, it is likely that these two mPRs are associated with the somatic cell membranes and mPRα is an intermediary of DHP action.

It was recently found that the mPRα protein is localized to the plasma membranes of both granulosa and theca cells that have been isolated from Atlantic croaker ovaries (Dressing et al., 2010). It was further demonstrated that endogenous mPR signaling in the ovarian follicle might suggest a role for mPRα in mediating the anti-apoptotic actions of progestins on ovarian follicle cells. mPRα may play a similar physiological role in the medaka ovary; the biological role of mPRγ however is unknown. We suggest that mPRγ may have a function that is distinct from that of mPRα given their different effects on the downstream reactions of the intracellular cAMP levels. Interestingly, the mRNA expression levels of *mPRγ*, but not *mPRα*, tend to increase as the time of ovulation approaches, suggesting that the ratio of mPRα to mPRγ may change at different times before ovulation. Such differential expression patterns of the two membrane progestin receptors may be indicative of their different roles in the follicles. In addition, as described above, large preovulatory follicles express two forms of *mPRγ* transcripts, indicating that mPRγ may exist as two distinct isoforms in the follicles. This further raises the question of whether the two mPRγ proteins play the same role in the follicles.

The present study was initiated to establish a basis for further examining whether mPRs associated with the granulosa cells of preovulatory follicles may have a role in medaka ovulation. We recently reported that nPR is a critical transcription factor for the expression of *EP4b* (Hagiwara et al., 2014), *plasminogen activator inhibitor-1* and *MT2-MMP* (Ogiwara et al., 2013; Ogiwara et al., 2015), all of which are presumed to be ovulation-related genes, in the medaka preovulatory follicles. Currently, studies investigating of the role of mPRs in nPR-directed expression of these genes in the follicular granulosa cells are underway.

In summary, the present study revealed the expression of *mPRα* and *mPRγ* in the granulosa cells of the ovulatory follicles of the medaka ovary. The in vitro activation of mPRα through DHP ligand binding decreased intracellular cAMP concentrations in HEK293T cells that expressed medaka recombinant mPRα. It is likely that mPRα expression associated with both the oocyte plasma membrane and the cell membranes of the granulosa cells may be simultaneously activated by DHP, which is abundant in the follicle approximately 12 h before ovulation. This study has established the basis for further investigations to clarify the role of granulosa cell-expressing mPRs in association with medaka ovulation.

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