Animal Reproduction Science 208 (2019) 106107

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Characterization, relative abundances of mRNA transcripts, and subcellular localization of two forms of membrane progestin receptors (mPRs) in the common Chinese cuttlefish, *Sepiella japonica*

Zan Pang[a], Zhenming Lü[a,b,*], Maoting Wang[a], Li Gong[a,b], Bingjian Liu[a,b], Lihua Jiang[a,b], Liqin Liu[a,b,**]

[a] National Engineering Laboratory of Marine Germplasm Resources Exploration and Utilization, College of Marine Science and Technology College, Zhejiang Ocean University, No. 1, South Haida Road, Dinghai District, Zhoushan, China
[b] National Engineering Research Center for Facilitated Marine Aquaculture, No. 1, South Haida Road, Dinghai District, Zhoushan, China

ARTICLE INFO

Keywords:
Cephalopod
Maturation
Gene expression
Progesterone receptor
Mollusc
Phylogeny

ABSTRACT

The membrane progestin receptor (*mPR*) family has been characterized in several species, including fish, frogs, rats, and humans. Results of previous studies indicate *mPRs* mediate the rapid, nongenomic action of progestins. In this study, the full-length cDNA of *Sepiella japonica mPR-beta* (*mPRβ*) and *mPR-gamma* (*mPRγ*) were characterized. Furthermore, *sjmPR* mRNA relative abundances were assessed for different tissues. There was also determination of the subcellular localization of *mPRs*, and investigation of the effect of *sjmPRs* on ovarian development via proximate actions on the brain and ovary of *S. japonica*. Results of tissue distribution assays indicated *mPRβ* and *mPRγ* transcripts were present predominantly in the brain and ovary. As ovaries developed, the abundance of *SjmPRs* mRNA transcripts increased and peaked during the interstitial growth phase (III), followed by a marked decrease afterward in both the brain and ovary. In addition, confocal microscopy evaluations of HEK293 T cells expressing the *mPRs*-EGFP gene indicated both *SjmPRβ* and *SjmPRγ* were localized in the plasma membrane of HEK293 T cells. Taken together, these findings indicate *S. japonica* protein is a membrane progestin receptor capable of inducing ovary maturation in cephalopods.

1. Introduction

The common Chinese cuttlefish, *Sepiella japonica*, is one of the most economically important cephalopod species, and is distributed widely throughout East Asia (Jerep and Roper, 2005). When managed in non-natural habitats, the sexual precocity of *S. japonica* results in cultivated individuals having a smaller adult body size than individuals in their natural habitat, a fact that has limited aquaculture development for this commercially important species (Wu et al., 2010). As a result, the molecular mechanisms regulating the sexual maturation of *S. japonica* have generated broad interest among researchers. The identification and characterization of differentially expressed genes active during ovarian development are an important initial approach for understanding the molecular regulation of reproductive maturation in *S. japonica*.

* Corresponding author.
** Corresponding author.
E-mail addresses: nblzmnb@163.com (Z. Lü), liuliqin-666@163.com (L. Liu).

https://doi.org/10.1016/j.anireprosci.2019.106107
Received 29 January 2019; Received in revised form 8 June 2019; Accepted 24 June 2019
Available online 25 June 2019
0378-4320/ © 2019 Published by Elsevier B.V.

Progesterone and its derivatives (progestins) are important steroid hormones that have been extracted in many invertebrates and have functions in many different physiological processes, including oocyte maturation, spermiogenesis, and the stimulation and inhibition of cell proliferation (Graham and Clarke, 1997; D'Aniello et al., 1996; Warrier et al., 2001; Custodia-lora et al., 2004; Meunpol et al., 2007; Yano, 1987; Rodríguez et al., 2002). Similar to other steroids, the effects of progestins are mediated via both classical genomic mechanisms and rapid, non-genomic mechanisms. Progestins exert this action through binding to nuclear progestin receptors (NPRs) as the classical pathway, which lead to modulation of gene transcription and translation activity (Mangelsdorf et al., 1995), and this biological response is relatively slow (Zhu et al., 2008). Progestins, however, have rapid actions via intracellular signaling transduction pathways within a few minutes (Edwards, 2005). Furthermore, microinjection of oocytes with progestins did not induce oocyte maturation (Lutz et al., 2000). These findings indicate such action is non-genomic and is mediated by binding to specific membrane progestin receptors (*mPRs*; Wang et al., 2014).

The *mPRs* have been identified and biochemically characterized in several species, including *Danio rerio* (Zhu et al., 2003a), *Anguilla anguilla* (Morini et al., 2017) and *Micropogonias undulatus* (Tubbs et al., 2010). The *mPRs* are highly expressed in various tissues and are involved in many specific actions, such as induction of oocyte maturation (Zhu et al., 2003a, b), stimulation of sperm hyper-motility (Thomas et al., 2005) and modulation of reproductive signaling in the brain (Ben-Yehoshua et al., 2007). Resumption of oocyte maturation is one of the most extensively studied and well-characterized non-genomic progestin actions and is mediated by *mPRs* via activation of G-protein-coupled signal transduction pathways (Thomas et al., 2007). The binding of progesterone to *mPRs* activates pertussis-sensitive inhibitory proteins ($G_{\alpha i}$) and induces decreases in adenylyl cyclase activity and intracellular cAMP concentrations, which in turn results in the activation of cAMP-dependent protein kinase A (PKA; Mehlmann et al., 2002). Inactivation of PKA stimulates the translation of mRNA encoding Mos and activates the mitogen-activated protein kinase (MAPK) pathway (Schmitt and Nebreda, 2002), which leads to germinal vesicle breakdown (GVBD) and oocyte maturation (Maller, 2001).

Although the non-genomic, rapid actions of progesterone have been studied in many species (Kazeto et al., 2005b; Morini et al., 2017; Kowalik et al., 2018), there are limited data where the functions of progestins and receptors have been assessed in ovarian development in invertebrates (Preechaphol et al., 2010). In *S. japonica*, to investigate the possible action of induction oocyte development in the present study, there was cloning and characterization of two *S. japonica mPRs* (*mPRβ* and *mPRγ*). In addition, there was examination of the relative abundances profiles of *mPRβ* and *mPRγ* mRNA in different tissues and different development stages in the brain and ovary of *S. japonica*. The subcellular localization of *mPRs* was also determined so as to ascertain the locations of this receptor in the cells of reproductive tissues of *S. japonica*.

2. Materials and methods

2.1. Animals

Adult cuttlefish (*S. japonica*) were captured from the aquaculture station of the Marine Fisheries Research Institute of Zhejiang on Xishan Island (Suzhou, China). Live cuttlefish were taken to the laboratory and sexed. Five adult cuttlefish of each sex were collected for tissue distribution analysis. Before tissue sample collection, cuttlefish were anesthetized in a solution of magnesium chloride (27.0 g/L). For tissue distribution analysis, samples of various tissues (*i.e.*, brain, liver, optic lobe, pancreas, muscle, heart, testis, ovary, and nidamental gland) were collected from both male and female *S. japonica* individuals using the procedure described by Chen (1991). These tissue samples were immediately frozen in liquid N_2 and kept at −70 ℃ until RNA extraction.

To evaluate patterns for relative abundances of *mPR* mRNA during the reproductive cycle, ovarian development was classified into four stages (I-IV) according to ovary diameter (Laptikhovsky and Arkhipkin, 2001; Luo et al., 2014). Five cuttlefish of each stage were collected. Liver and ovary tissue samples were taken at four different stages of development and were stored in liquid N_2 until needed.

2.2. Isolation of RNA and reverse transcription PCR (RT-PCR)

Total RNA was isolated from *S. japonica* tissues using Trizol (Invitrogen, USA) using a previously published protocol (Lü et al., 2016). Genomic DNA was treated with DNase I (TaKaRa, Japan) at 37 ℃ for 30 min to reduce the possibility of gDNA contamination. The RNA quantity and purity were assessed using a NanoDrop spectrophotometer (Thermo Electron Corporation, USA) followed by gel electrophoresis on a 0.8% agarose gel. Two micrograms of extracted RNA were used to synthesize cDNA using M-mlV RTase cDNA Synthesis Kits (TaKaRa, Japan) according to the manufacturer's instructions.

2.3. Cloning of cDNA for different mPR subtypes from cuttlefish ovary tissue

To clone partial fragments of *S. japonica mPR*, primer pairs were designed based on nucleotide sequences obtained from prior studies of the *S. japonica* ovary transcriptome. The PCR amplification of *mPR* segments was performed using these primers along with a cDNA template and Ex Taq Mix (CWBIO, China). Amplification was conducted using the following conditions: 94 ˚C for 3 min, 35 cycles of 94 ˚C for 30 s, 61 ℃/65 ℃ for 30 s, and 72 ˚C for 90 s; and a final extension at 72 ˚C for 10 min. The resulting PCR products were inserted into pMD™18-T Vectors (TaKaRa, Japan) and were sequenced.

Subsequently, the 3′ and 5′-regions were amplified using a 3′-Full RACE Core Set with PrimeScript™ RTase and a SMARTerRACE 5′ Kit with TAP. Rapid amplification of cDNA ends-PCR (RACE-PCR) was performed according to the manufacturer's instructions. All gene-specific primers used in this study are listed in Table 1. Amplified products were cloned into a pGEM-T Easy vector (TaKaRa,

Table 1
Primers used in this text.

Primer name	Sequence (5'-3')	Usage
MPRβ F	AAACCGACTGTCACCAAGGA	*MPRβ* cDNA fragment cloning
MPRβ R	CTAATGTCAGGGAGGAGGAT	
3'R- *MPRβ*1	CGTTATAAGCGACCCTATCCAT	*MPRβ* 3'-RACE cloning
3'R- *MPRβ*2	GCAGGTTTCTTCTTTGGTTCAG	
5'R- *MPRβ*1	TGGTACTTGCTCCTTGGTGACA	*MPRβ* 5'-RACE cloning
5'R- *MPRβ*2	AGCGAGTCTGTTGTTGTGGTTG	
MPRγ F	CGCAGAGAGCAAGCCACACCAT	*MPRγ* cDNA fragment cloning
MPRγ R	CCGCCGCTACTTTTAAACGCAA	
3'R- *MPRγ*1	TCTTGCTTATCTCTTGGCCTGC	*MPRγ* 3'-RACE cloning
3'R- *MPRγ*2	GAACGCACGTGGTTTGGATACT	
5'R- *MPRγ*1	GCCAAGAGATAAGCAAGAAATG	*MPRγ* 5'-RACE cloning
5'R- *MPRγ*2	GGAAGGAAGTGAGTCCAGAAGT	
q-*MPRβ* F	TCCGACATCTGAACCAACCA	*MPRβ* qPCR
q-*MPRβ* R	CAACACCCAGTCCGATCCCT	
q-*MPRγ* F	TGCGTATTGCAGTCTTTCTTGA	*MPRγ* qPCR
q-*MPRγ* R	ACGGTGGCCTTAGTTTGTTTAG	
β-actin F	GCCAGTTGCTCGTTACAG	β-actin qPCR as internal reference
β-actin R	GCCAACAATAGATGGGAAT	

Japan) and sequenced. Sequence data was analyzed using GENETYX version 14.11 (Genetyx, Tokyo, Japan).

2.4. Structural and phylogenetic analysis

Predicted amino acid sequences and protein size were determined using the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weights of deduced *mPR* proteins were determined using ExPASy ProtParam (http://www.expasy.org/tools/protparam.html). The NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) was used to identify phosphorylation sites. N-glycosylation sites were identified by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). A homology search for amino acid sequences was conducted using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were conducted using ClustalW, and bootstrapped phylogenetic trees were constructed using the neighbor-joining (NJ) method implemented in MEGA7.0 (Saitou and Nei, 1987).

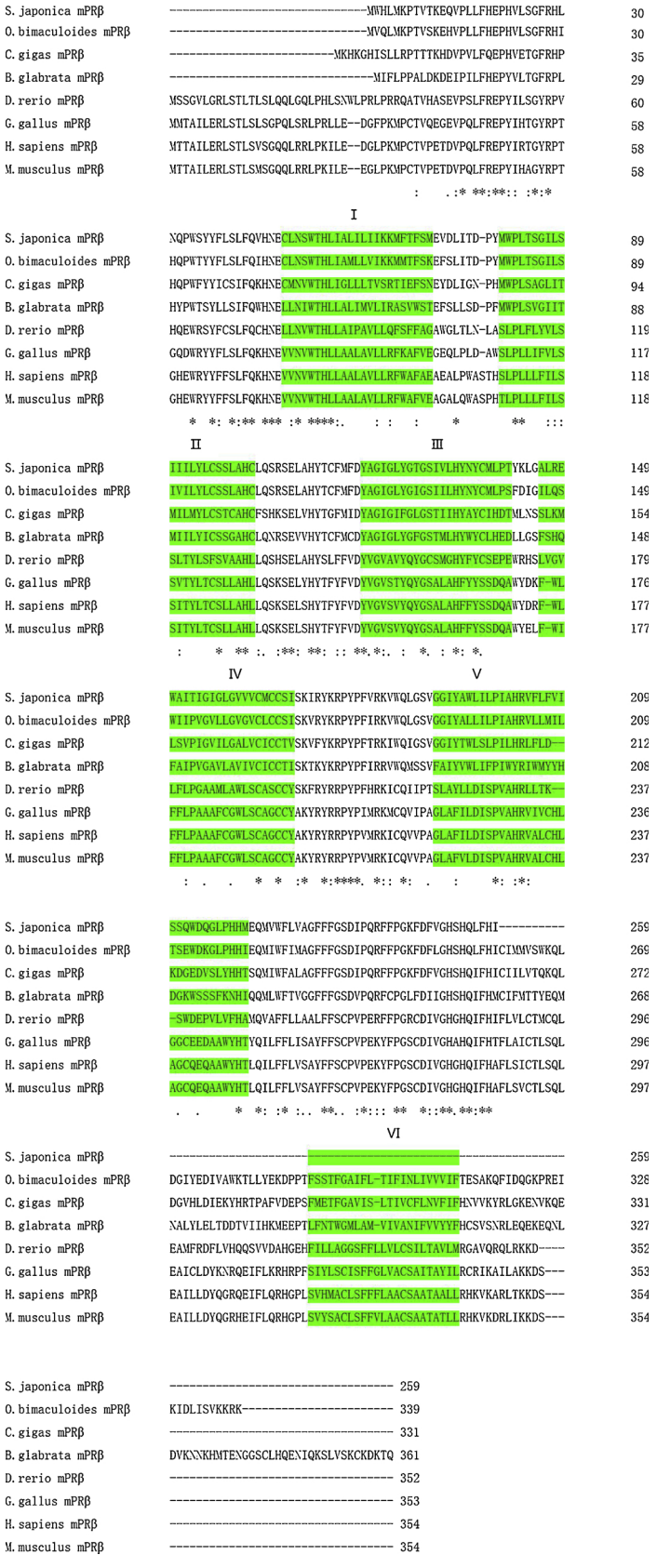
2.5. Expression of S. japonica mPRs in different tissues and at different development stages

The relative abundances of *mPR* mRNA in different tissues (i.e., brain, liver, optic lobe, pancreas, muscle, heart, and ovary tissue, as well as the nidamental gland of female cuttlefish and the testis of male cuttlefish) and in brain and ovary tissues at different development stages (i.e. stages I, II, III, and IV) were assessed using real-time quantitative PCR (qRT-PCR). Total RNA was isolated using a Trizol (TaKaRa, Japan) protocol. After extraction, total RNA was primed with a clamped oligo (dT) primer and was reverse-transcribed using M-MLV reverse transcriptase (TaKaRa, Japan). The qRT-PCR reaction solution (at a total volume of 20 μL) contained 0.8 μL cDNA template, 0.8 μL of both forward and reverse primers (10 μM), 10 μL of SYBR® Premix Ex Taq™II (TaKaRa, Japan), 0.4 μL ROX Reference Dye II (TaKaRa, Japan), and 7.2 μL RNase-free water. Reaction conditions included an initial incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension of 5 min at 72 °C. The β-actin gene of *S. japonica* (accession no. JN564496.1) was used as a reference and all samples were run in triplicate to ensure accuracy and reproducibility (He et al., 2014). Relative abundances of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method (Livak and Schmittgen, 2001).

2.6. Subcellular localization of S. japonica mPRs

There was determination of subcellular localization of putative *S. japonica mPRs* by constructing fusion proteins that included enhanced green fluorescent protein (EGFP) tags. Primer pairs were designed for the pEGFP-N1 plasmid based on full-length cDNA sequences to amplify the complete ORF for *mPRs*. The PCR products were cloned and ligated into a vector using a Rapid DNA Ligation Kit. The recombinant plasmid was named pEGFP-N1-*mPR* and was extracted using an Endofree plasmid KIT II (Tiangen, Beijing, China) according to the manufacturer's instructions. The identity of the fusion cDNA was confirmed by sequencing.

To determine whether *mPRs* were present in the cell membrane, cells from human embryonic kidney cell line (HEK293 T) were maintained in Dulbecco modified Eagle medium (DMEM, supplemented with 10% fetal bovine serum, 4 mM l-glutamin and100U/mL penicillin-streptomycin) under humidified conditions with 5% CO_2 at 37 ℃. The *mPRs*-EGFP plasmid constructs were transfected into HEK293 T cells using Lipofectamine 2000 (Invitrogen, Madison, WI, USA) according to the manufacturer's instructions. 24 h after transfection, HEK293 T cells stably expressing the *mPRs*-EGFP construct were washed with PBS, then were fixed with 4% paraformaldehyde for 20–30 min at room temperature. Next, cells were again washed with PBS, incubated with the membrane probe DiI (Beyotime, Haimen, China) for 20 min at room temperature and protected from light. Cells were then washed with PBS and stained



(caption on next page)

Fig. 1. Multiple alignment of the deduced amino acid sequence of *mPR*β from *Sepiella japonica* with the *mPR*β proteins of other species; Asterisks (*) denote the presence of the same amino acids in different sequences; and a period (.) indicates the extent of conservation of different amino acids; Transmembrane domains of *mPR*βs are highlighted in green; Accession numbers of sequences used in the figure are included below: *S. japonica* mPRβ (AVA07271.1), *O. bimaculoides* mPRβ (XP_014774875.1), *C. gigas* mPRβ (XP_019918893.1), *B. glabrata* mPRβ (XP_013077721.1), *D. rerio* mPRβ (NP_899187.1), *G. gallus* mPRβ (NP_001008462.1), *H. sapiens* mPRβ (NP_588608.1), *M. musculus* mPRβ (NP_083105.3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with DAPI (Beyotime, Haimen, China) for 5 min at room temperature and protected from light. Cells were visualized using a confocal laser-scanning microscope (Leica TCSSP5, Germany).

3. Results

3.1. Isolation and characterization of cDNA clones of cuttlefish mPRs

Two *mPR* cDNAs were obtained by utilizing 5′and 3′-RACE using gene-specific primers; these were designated *SjmPRβ* (accession no. KY709673.2) and *SjmPR*γ (accession no. KY709674.2). The full-length cDNAs of *SjmPRβ* and *SjmPR*γ were 1876 and 1364 bp in length, respectively, and the coding regions of the β and γ forms were 780 and 1050 bp in length. These cDNA encoded proteins 249 and 349 amino acids in length with predicted molecular masses of 30.07 and 40.91 KDa, respectively. Multiple sequence alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) indicated that *SjmPRβ* had little amino acid sequence identity (30.38%) with *SjmPR*γ.

There were several structural motifs identified. These included: An N-glycosylation site (N{P}S/T{P}) (78–81 residues) that was present only in *SjmPR*γ, and a phosphorylation sites in both subtypes (*SjmPRβ*:16; *SjmPR*γ: 27). Use of a computer analyses of deduced amino acid sequences by SOSUI (http://sosui.proteome. bio.tuat.ac.jp/sosuiframe0.html) resulted in the prediction that the *SjmPR*γ protein has seven transmembrane domains and was located in the plasma membrane, which is characteristic of G protein-coupled membrane receptors. The use of the same software, however, provided information for the prediction that the *SjmPRβ* protein should have five transmembrane domains.

The amino acid sequences of each *SjmPR* and related forms in other species are aligned in Figs. 1 and 2. The N-terminal region was highly conserved between *SjmPR*γ and other *mPR*s, and the C-terminal region was highly conserved between *SjmPRβ* and other *mPR*s. The deduced amino acid sequence of *SjmPRβ* had considerable identity to *mPRβs* present in other mollusks (55%–82%) and 37%–41% sequence identity with forms found in chordates. The *SjmPR*γ protein was homologous to the forms of *mPR*γ found in *Octopus bimaculoides* (54%), *Biomphalaria glabrata* (47%), *Aplysia californica* (43%), *Homo sapiens* (41%), *Mus musculus* (40%), *Oryzias latipes* (41%), and *Gallus gallus* (39%).

A phylogenic tree was constructed using the neighbor-joining method based on *mPR* amino acid sequences (mPRα, mPRβ, mPRγ and mPRδ) from various vertebrate and invertebrate species (Fig. 3). The phylogeny was divided into two major monophyletic groups: the first comprising mPRα and mPRβ from vertebrate and invertebrate, and the second clustering of two additional isoforms (mPRγ and mPRδ) from vertebrate and invertebrate. The *S. japonica* mPRβ and mPRγ clustered together with respective mPR types among invertebrate representatives and were especially close to *Octopus bimaculoides*.

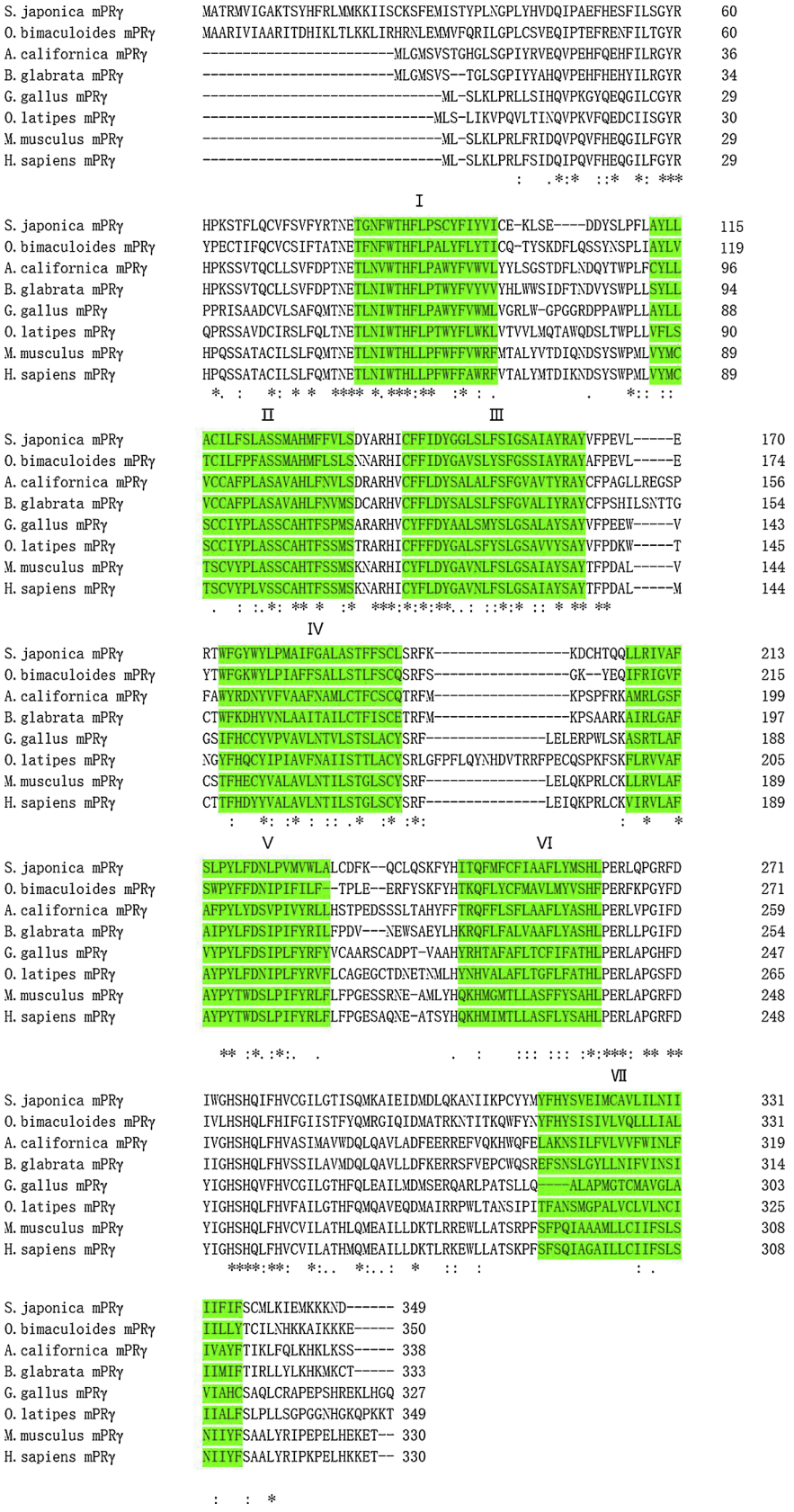
3.2. Tissue distribution of S. japonica mPRs

To detect the relative abundance profiles of *SjmPRβ* and *SjmPR*γ mRNA transcripts, qRT-PCR was conducted using cDNA isolated from adult female and male *S. japonica* tissues, including the brain, liver, muscle, heart, pancreas, optic lobe, ovary, nidamental gland, and testis, using β-actin as the internal reference. The results indicated the abundance of β-actin mRNA was similar in all examined tissues.

Both transcripts were present in all tissues. The *SjmPRβ* and *SjmPR*γ mRNA transcripts were in large abundances in the brain, ovary, nidamental gland, and testis. There were lesser relative abundances of these mRNA transcripts in other tissues (Fig. 4A and B). The relative abundances of *SjmPR*γ mRNA transcripts in the brain were greater than in the ovary, however, there were no significant differences between the relative abundance of *SjmPRβ* mRNA in brain and ovarian tissues ($P > 0.05$).

3.3. Relative mRNA abundance profiles of S. japonica mPRs during ovary development

Because *SjmPRβ* and *SjmPR*γ mRNA were highly expressed in the brain and ovary, there was examination of the mRNA relative abundance profiles of *SjmPR*s during ovarian development only in brain and ovary tissue. In general, for *SjmPRβ* and *SjmPR*γ there were similar mRNA relative abundance patterns (Fig. 5A and B). The relative abundances of mRNA transcripts of both *SjmPR*s increased as the ovaries developed from stage I to III and reached peak relative abundances at Stage III in both brain and ovary tissue. Furthermore, the relative abundance of *mPR* was markedly less at stage IV. The relative abundances of both *SjmPR* mRNA transcripts were less in brain than in ovarian tissue at Stages II and III ($P < 0.05$).



(caption on next page)

Fig. 2. Multiple alignment of the deduced amino acid sequence of *mPRγ* from *Sepiella japonica* with the *mPRγ* proteins of other species; Asterisks (*) denote the presence of the same amino acids in different sequences; and a period (.) indicates the degree of conservation among different amino acids; Transmembrane domains of *mPRγ*s are highlighted in green; Accession numbers of sequences used in the figure are included below: *S. japonica* mPRγ (AVA07272.1), *O. bimaculoides* mPRγ (XP_014787281.1), *A. californica* mPRγ (XP_005103208.1), *B. glabrata* mPRγ (XP_013087777.1), *G. gallus* mPRγ (XP_015147701.2), *O. bimaculoides* mPRγ (XP_014787281.1), *M. musculus* mPRγ (NP_083024.1), *H. sapiens* mPRγ (NP_060175.3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

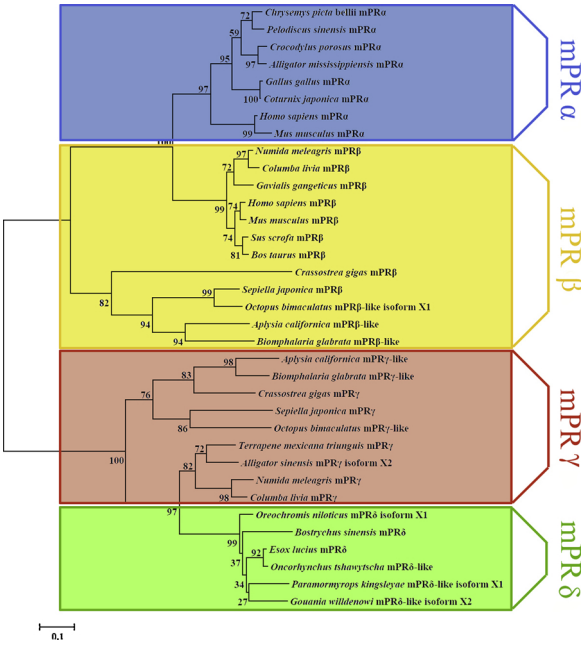


Fig. 3. Phylogenetic analysis of *SjmPRβ* and *SjmPRγ*. A phylogenetic tree was constructed using the neighbor-joining method; Number shown at each branch node indicates the bootstrap value (%).

3.4. Subcellular localization of S. japonica mPRs

To determine the subcellular localization of *S. japonica mPR*s, there was localization of C-terminal EGFP-tagged *S. japonica mPR*s in HEK293 T cells. There was a large amount of specific fluorescence in transfected cells when assessment occurred confocal fluorescence microscopy. Fluorescence was mainly detected in the plasma membrane (Fig. 6), which indicated both forms were exclusively localized in the cell membrane, which may indicate that both forms are membrane receptors.

4. Discussion

Because cDNAs encoding *mPR*s were first identified in *Cynoscion nebulosis* (spotted seatrout), many *mPR*s have been characterized in other species (Zhu et al., 2003a; Tokumoto et al., 2006; Thomas, 2004). Little, however, is known regarding *mPR* gene expression in mollusks.

In the present study, two *mPR*s (*SjmPRβ* and *SjmPRγ*) were first isolated from the ovarian tissue of *S. japonica*. These *S. japonica mPR* subtypes had a large amount of sequence identity with *mPR*s from other molluscan species (54%–82%). The *SjmPRβ* protein, however had a significant divergence from *SjmPRγ* in amino acid sequence, with an identity of approximately 30%. The results from the phylogeny analysis in the present study indicated vertebrate mPRβ first clustered with the mPRα clade, and then clustered together with mollusc mPRβ. This indicated that the clade of vertebrate mPRβ sequences are more related to vertebrate mPRα sequences than to mollusk mPRβ sequences. In the European eel, the mPRα and mPRβ isforms clustered with one monophyletic group (Morini et al., 2017). Furthermore, phylogenetic analyses clearly indicated there was a greater divergence between mPRγ clusters compared to the clusters of the α and β forms (Kazeto et al., 2005a; Morini et al., 2017).

The predicted structure of the *SjmPRγ* protein included seven transmembrane domains, making this protein similar to previously identified *mPRγs*. This evidence also supports the hypothesis that this protein is a G protein-coupled receptor (GPCR) (Tokumoto et al., 2012). In addition, *mPRβ* proteins generally have seven transmembrane domains, as occurs in goldfish (Tokumoto et al., 2012) and seatrout (Zhu et al., 2003a). The predicted structure, however, indicated *SjmPRβ* had five transmembrane domains, and this finding corroborated previous findings (Cserzo et al., 1997). An N-glycosylation site (N {P} S/T {P}; 78–81 residues) was reported to be present only in *SjmPRγ* but was also conserved in all *mPR* subtypes in goldfish (Tokumoto et al., 2012). Conserved regions in the γ form may be involved in ligand binding and other G-protein functions (Kazeto et al., 2005a).

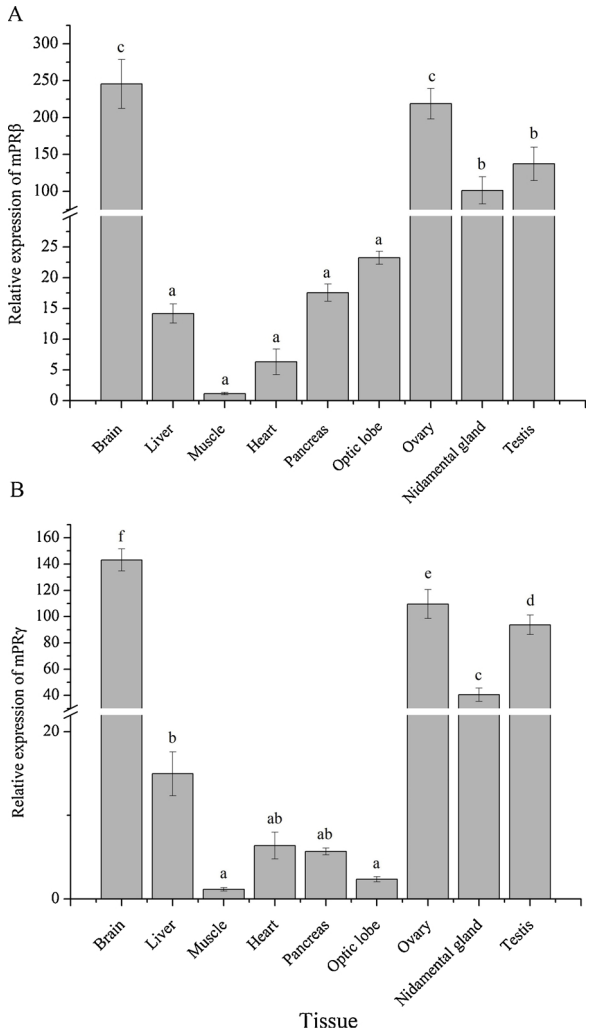


Fig. 4. Tissue-specific expression patterns of *SjmPRβ* and *SjmPR*γ; β-actin gene abundance was used as an internal control; Symbols and vertical bars represent the mean ± SD ($n = 6$): Different lowercase letters above the bars indicate significant differences ($P < 0.05$) between different tissues; Panels are included to depict results for mPRβ (A) and mPRγ (B).

The *mPR*s, which are primarily responsible for mediating the (non-genomic) action of progestins, are localized to reproductive tissues and the central nervous system (Ashley et al., 2006; O'Byrne et al., 1991; Kasubuchi et al., 2017). In the present study, *SjmPRβ* and *SjmPR*γ mRNA was present in all tissues investigated, but most prominently in brain and ovarian tissues. These tissues are targets of progesterone being an important regulator of reproduction. With respect to the localization of *SjmPR* mRNA, results are similar to those previously reported for other species (Kazeto et al., 2005a, b; Hanna and Zhu, 2009; Morini et al., 2017). Large abundances of *SjmPR* mRNA transcript in brain and ovarian tissues are consistent with *SjmPRs* having an important function in oocyte maturation (OM). The OM may be induced by *mPR*-mediated maturation inducing hormone (MIH) signaling. The MIH is secreted from ovarian follicle cells with the secretion being regulated by gonadotropins released from the pituitary gland (Nagahama, 1987), and may be involved in stimulating the *de novo* synthesis of cyclin B, a regulatory subunit of maturation promoting factor (MPF), the final mediator of oocyte maturation (Nagahama and Yamashita, 2008). Furthermore, the *mPR*-mediated progestin induction of OM in fish involves a decrease in intracellular cAMP, which involves the activation of a pertussis toxin-sensitive inhibitory G protein (Patino and Thomas, 1990).

The functions of *mPRs* in the *S. japonica* brain remain unclear, but findings in the present study are similar to those of previous studies of goldfish (Tokumoto et al., 2006) and spotted seatrout (Zhu et al., 2003a). Non-genomic actions of progesterone have also been reported in the brain (Tischkau and Ramirez, 1993). The greater abundance of *mPR* mRNA transcripts in brain indicate progesterone and its metabolites may also have non-genomic functions in these neural tissues, possibly functioning with specific *mPRs* in binding to modulatory sites on neurotransmitter receptors such as the $GABA_a$/benzodiazepine receptor Cl^- channel complex (Lan et al., 1991; McEwen, 1991). Furthermore, both *mPRβ* and *mPR*γ were detected in various tissues of different species, indicating both genes are active in various tissues. The pig *mPRβ* gene was also expressed in cumulus cells, where it is involved in the regulation of *in*

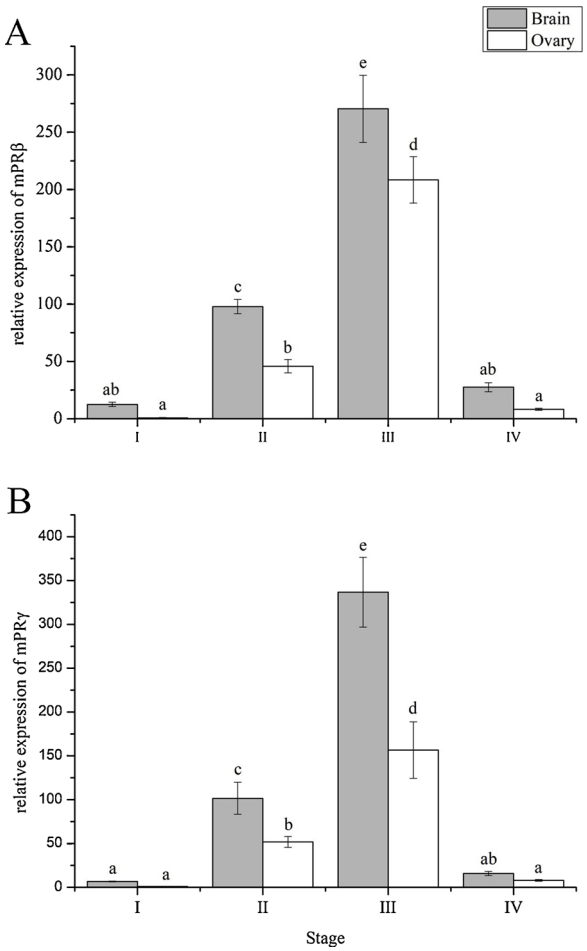


Fig. 5. Relative abundance of *SjmPRs* mRNA transcripts in brain and ovarian tissues at different ovarian development stages; β-actin abundance was used as an internal control; Symbols and vertical bars represent the mean ± SD ($n = 6$); Different lowercase letters above the bars indicate differences ($P < 0.05$) between different tissues; Panels are used to depict results for mPRβ (A) and mPRγ (B).

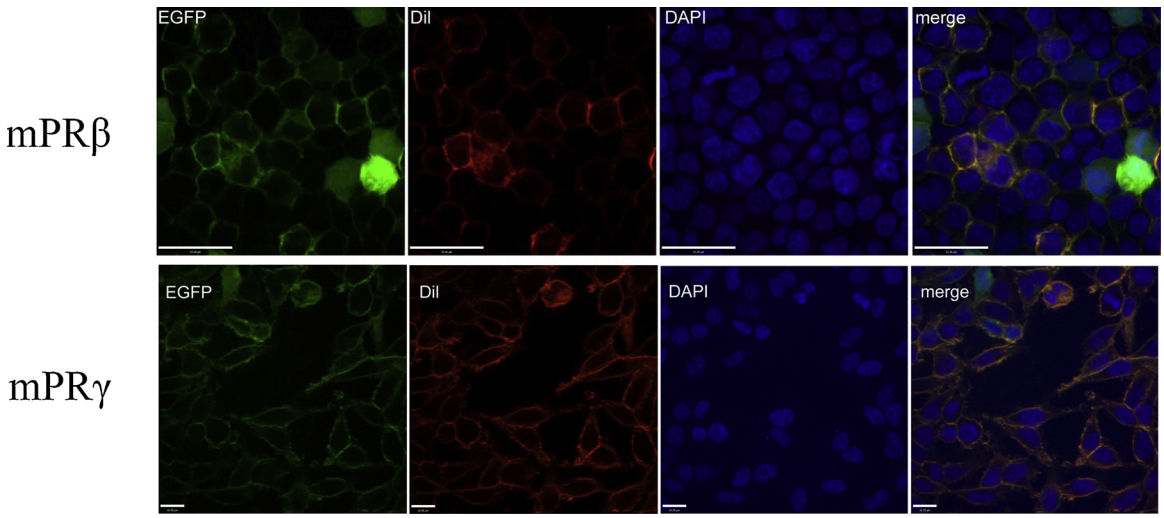


Fig. 6. Subcellular localization of SjmPRβ and SjmPRγ in HEK293 T cells; Left panel depicts the presence of mPR-EGFP; Cells were stained with a membrane plasma probe (DiI) and a nuclei probe (DAPI); Merged pictures are shown in the right panel Localization of proteins is depicted in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

vitro maturation of cumulus-oocyte complexes (Qiu et al., 2008). In *Xenopus*, *mPRβ* there is a progesterone receptor that is present in oocytes (Kazeto et al., 2005a), which indicates the *mPRβ* protein possesses a conserved structure for binding progesterone (Tokumoto et al., 2012). In channel catfish, the Ip*mPRγ* transcript was detected in gill, ventral aorta, intestine, and trunk kidney tissues, which indicates there are functions of this protein in ion regulation (Kazeto et al., 2005a). Taken together, these results indicate that variation in the tissue-specific distribution of different *mPRs* is related to the functional or species-specific differences.

During *S. japonica* female ovarian development, mRNAs for two *mPR* subtypes (*SjmPRβ* and *SjmPRγ*) were consistently present in large abundances. Changes in the relative abundances of *SjmPRβ* and *SjmPRγ* mRNA during ovarian maturation have been reported independently. The relative abundances increased from the oogonia phase (I) with subsequent developments, peaking in the interstitial growth phase (III). Findings in the present study closely paralleled the changes in abundances of *mPR* mRNA in *Sus scrofa* domestica (Diaz and Wiltbank, 2005). Furthermore, relative abundance of *SjmPR* mRNA peaked prior to ovarian maturation, which indicates there is a function of this protein in ovarian development (Maller, 1998).

In previous studies, there was inconsistent results concerning *mPR* subcellular localization (Qiu et al., 2008). Zhu et al. (2003b) reported that in spotted seatrout *mPRs* were localized to the plasma membrane. The *mPRs*, however, were localized in the endoplasmic reticulum, both in humans (Krietsch et al., 2006) and sheep (Ashley et al., 2006). One interesting finding was that pig *mPRβ* was localized in the plasma membrane of cumulus cells, but in MEFs, it was localized in the endoplasmic reticulum (Qiu et al., 2008). These inconsistencies are likely related to whether the N or C-terminus of the protein is exposed on the cell surface (Krietsch et al., 2006).

In the present study, both the *SjmPRβ* and *SjmPRγ* protein were identified by confocal fluorescence microscopy to be localized in the plasma membrane of HEK293 T cells. This may be due to the fact that *SjmPRs* span the membrane an odd number of times (β: 5; γ: 7) so that the terminus is located outside the cell.

In summary, in the present study *mPR* (*i.e.*, *mPRβ* and *mPRγ*) mRNA was in a large abundance in the major reproductive tissue of *S. japonica*, including in the brain and ovary. Both *SjmPRβ* and *SjmPRγ* were localized in the plasma membrane of HEK293 T cells. Furthermore, there was a marked increase in *SjmPR* abundance in the brain and in the ovary when there was oocyte maturation occurring and there was a subsequent decrease in trophoplasmic growth. Data from the present study indicated that in *S. japonica*, two distinct *mPRs* mediated a number of progestin-regulated non-genomic actions, particularly oocyte maturation. Thus, results of the present study provide new insights into the regulation of ovary development in cephalopods. Further studies including hormonal regulation and intracellular signaling relevant to cephalopod ovary development are required to identify conserved mechanisms and pathways during this developmental period.

Declaration of Competing Interest

The authors of this manuscript certify that they have no conflicts of interest to declare.

Acknowledgments

This research was supported by the Natural Science Foundation of China (41406138), the Open Project of Zhejiang Provincial Top Key Discipline of Biotechnology (KF2016008), and a Project of the Bureau of Science and Technology of Zhoushan (2014C41007).

References

Ashley, R.L., Clay, C.M., Farmerie, T.A., Niswender, G.D., Nett, T.M., 2006. Cloning and characterization of an ovine intracellular seven transmembrane receptor for progesterone that mediates calcium mobilization. Endocrinology 147, 4151–4159. https://doi.org/10.1210/en.2006-0002.

Ben-Yehoshua, L.F., Lewellyn, A.L., Thomas, P., Maller, J.L., 2007. The role of Xenopus membrane progesterone receptor β in mediating the effect of progesterone on oocyte maturation. Mol. Endocrinol. 21, 664–673. https://doi.org/10.1210/me.2006-0256.

Chen, H.Y., 1991. The preparation of the specimen of the reproductive system of squid. J. Lishui Teachers' College 1 67-67. (in Chinese).

Cserzo, M., Wallin, E., Simon, I., Heijne, G., Elofsson, A., 1997. Prediction of transmembrane a-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng. 10, 673–676. https://doi.org/10.1093/protein/10.6.673.

Custodia-lora, N., Novillo, A., Callard, I., 2004. Effect of gonadal steroids on progesterone receptor, estrogen receptor, and vitellogenin expression in Male Turtles (*Chrysemys picta*). J. Exp. Zool. A. Comp. Exp. Biol. 301, 15–25. https://doi.org/10.1002/jez.a.20004.

Mangelsdorf, D.F., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839. http://linkinghub.elsevier.com/retrieve/pii/009286749590199X.

D'Aniello, A., Di Cosmo, A., Di Cristo, C., Assisi, L., Botte, V., Di Fiore, M.M., 1996. Occurrence of sex steroid hormones and their binding proteins in *Octopus vulgaris* lam. Biochem. Biophys. Res. Commun. 227, 782–788. https://doi.org/10.1006/bbrc.1996.1585.

Diaz, F.J., Wiltbank, M.C., 2005. Acquisition of luteolytic capacity: changes in prostaglandin $F_{2\alpha}$ regulation of steroid hormone receptors and estradiol biosynthesis in pig corpora lutea. Biol. Reprod. 28, 172–189. https://doi.org/10.1095/biolreprod.103.020461.

Edwards, D.P., 2005. Regulation of signal transduction pathways by estrogen and progesterone. Annu. Rev. Physiol. 67, 335–376. https://doi.org/10.1146/annurev.physiol.67.040403.120151.

Graham, J.D., Clarke, C.L., 1997. Physiological action of progesterone in target tissues. Endocr. Rev. 18, 502–519. https://doi.org/10.1210/edrv.18.4.0308.

Hanna, R.N., Zhu, Y., 2009. Expression of membrane progestin receptors in zebrafish (*Danio rerio*) oocytes, testis and pituitary. Gen. Comp. Endocrinol. 161, 153–157. https://doi.org/10.1016/j.ygcen.2008.10.006.

He, J.Y., Chi, C.F., Liu, H.H., 2014. Identification and analysis of an intracellular Cu/Zn superoxide dismutase from Sepiella maindroni under stress of Vibrio harveyi and Cd^{2+}. Dev. Comp. Immunol. 47, 1–5.

Jerep, P., Roper, C.F.E., 2005. Cephalopods of the world. An annotated and illustrated catalogue of cephalopod species known to date. Vol. 1. Chambered Nautiluses and Sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae and Spirulidae). FAO Species Catalogue for Fishery Purposes Vol. 1 FAO, Rome No 4.

Kasubuchi, M., Watanabe, K., Hirano, K., Inoue, D., Li, X., Terasawa, K., Konishi, M., Itoh, N., Kimura, I., 2017. Membrane progesterone receptor beta (mPRβ/Paqr8) promotes progesterone-dependent neurite outgrowth in PC12 neuronal cells via non-G protein-coupled receptor (GPCR) signaling. Sci. Rep. 7, 5168. https://doi.org/10.1038/s41598-017-05423-9.

Kazeto, Y., Goto-Kazeto, R., Thomas, P., Trant, J.M., 2005a. Molecular characterization of three forms of putative membrane-bound progestin receptors and their tissue-distribution in channel catfish, Ictaluruspunctatus. J. Mol. Endocrinol. 34, 781–791. https://doi.org/10.1677/jme.1.01721.
Kazeto, Y., Goto-Kazeto, R., Trant, J.M., 2005b. Membrane-bound progestin receptors in channel catfish and zebrafish ovary: changes in gene expression associated with the reproductive cycles and hormonal reagents. Gen. Comp. Endocrinol. 142, 204–211. https://doi.org/10.1016/j.ygcen.2005.01.017.
Kowalik, M.K., Rekawiecki, R., Kotwica, J., 2018. Expression of membrane progestin receptors (mPRs) in the bovine corpus luteum during the estrous cycle and first trimester of pregnancy. Domest. Anim. Endocrinol. 63, 69–76. https://doi.org/10.1016/j.domaniend.2017.12.004.
Krietsch, T., Fernandes, M.S., Kero, J., Losel, R., Heyens, M., Lam, E.W.-F., Huhtaniemi, I., Brosens, J.J., Gellersen, B., 2006. Human homologs of the putative G protein-coupled membrane progestin receptors (mPRalpha, beta, and gamma) localize to the endoplasmic reticulum and are not activated by progesterone. Mol. Endocrinol. 20, 3146–3164. https://doi.org/10.1210/me.2006-0129.
Lan, N.C., Bolger, M.B., Gee, K.W., 1991. Identification and characterization of a pregnane steroid recognition site that is functionally coupled to an expressed GABAA receptor. Neurochem. Res. 16, 347–356. https://doi.org/10.1007/BF00966098.
Laptikhovsky, V.V., Arkhipkin, A.I., 2001. Oogenesis and gonad development in the cold water loliginid squid *Loligo gahi (*Cephalopoda: myopsida) on the Falkland shelf. J. Mollus. Stud 67, 475–482. https://doi.org/10.1093/mollus/67.4.475.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
Luo, J., Jiang, X.M., Liu, M.H., Tang, F., Peng, R.B., 2014. Oogenesis and ovarian development in Sepia lycidas. Acta Hydrobiol. Sin. 38, 1107–1116 (in Chinese).
Lutz, L.B., KimB, J.D., Hammes, S.R., 2000. G protein beta gamma subunits inhibit nongenomic progesterone-induced signaling and maturation in *xenopuslaevis* oocytes EVIDENCE FOR a RELEASE OF INHIBITION MECHANISM FOR CELL CYCLE PROGRESSION. J. Biol. Chem. 275, 41512–41520. https://doi.org/10.1074/jbc.M006757200.
Lü, Z.M., Liu, W., Liu, L.Q., Wang, T.M., Shi, H.L., Ping, H.L., Chi, C.F., Yang, J.W., Wu, C.W., 2016. Cloning, characterization, and expression profile of estrogen receptor in common chinese Cuttlefish. J. Exp. Zool. A. Ecol. Gen. Physiol 325, 181–193. https://doi.org/10.1002/jez.2011.
Maller, J.L., 2001. The elusive progesterone receptor in *Xenopus* oocytes. Proc. Natl. Acad. Sci. 98, 8–10. https://doi.org/10.1073/pnas.98.1.8.
Maller, J.L., 1998. Recurring themes in oocyte maturation. Biol. Cell 90, 453–460. https://doi.org/10.1111/j.1768-322X.1998.tb01054.x.
Mehlmann, L.M., Jones, T.L., Jaffe, L.A., 2002. Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. Science 297, 1343–1345.
McEwen, B.S., 1991. Non-genomic and genomic effects of steroids on neural activity. Trends Pharmacol. Sci. 12, 141–147. https://doi.org/10.1126/science.1073978.
Morini, M., Peñaranda, D.S., Vílchez, M.C., Nourizadeh-Lillabadi, R., Lafont, A.G., Dufour, S., Asturiano, J.F., Weltzien, F., Pérez, L., 2017. Nuclear and membrane progestin receptors in the European eel: characterization and expression *in vivo* throughspermatogenesis. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 207, 79–92. https://doi.org/10.1016/j.cbpa.2017.02.009.
Meunpol, O., Pai, S., Suthikrai, W., Piyatiratitivorakul, S., 2007. Identification of progesterone and 17α-hydroxyprogesterone in polychaetes (Perinereis sp.) and the effects of hormone extracts on penaeid oocyte development *in vitro*. Aquaculture 270, 485–492. https://doi.org/10.1016/j.aquaculture.2007.05.031.
Nagahama, Y., 1987. Gonadotropin action on gametogenesis in teleost gonads. Zool. Sci. 4, 209–222.
Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Dev. Growth Differ. 50, S195–219. https://doi.org/10.1111/j.1440-169X.2008.01019.x.
O'Byrne, K.T., Thalabard, J.C., Grosser, P.M., Wilson, R.C., Williams, C.L., Chen, M.D., Ladendorf, D., Hotchkiss, J., Knobil, E., 1991. Radiotelemetric monitoring of hypothalamic gonadotropin-releasing hormone pulse generator activity throughout the menstrual cycle of therhesus monkey. Endocrinology 129, 1207–1214. https://doi.org/10.1210/endo-129-3-1207.
Patino, R., Thomas, P., 1990. Characterization of membrane receptor activity for 17α, 20β, 21-trihydroxy-4-pregnen-3-one in ovaries of *spotted seatrout* (*Cynoscion nebulosus*). Gen. Comp. Endocr. 78, 204–217. https://doi.org/10.1529/biophysj.103.035527.
Preechaphol, R., Klinbunga, S., Yamano, K., Menasveta, P., 2010. Molecular cloning and expression of progestin membrane receptor component 1(Pgmrc1) of the giant tiger shrimp Penaeus monodon. Gen. Comp. Endocrinol. 168, 440–449. https://doi.org/10.1016/j.ygcen.2010.06.002.
Qiu, H.B., Lu, S.S., Ji, K.L., Song, X.M., Lu, Y.Q., Zhang, M., Lu, K.H., 2008. Membrane progestin receptor beta (mPR-β): a protein related to cumulus expansion that is involved in *in vitro* maturation of pig cumulus oocyte complexes. Steroids 73, 1416–1423. https://doi.org/10.1016/j.steroids.2008.07.007.
Rodríguez, E.M., Medesani, D.A., Greco, L.S.L., Fingerman, M., 2002. Effects of some steroids and other compounds on ovarian growth of the red swamp crayfish, *Procambarus clarkii*, during early vitellogenesis. J. Exp. Zool. 292, 82–87. https://doi.org/10.1002/jez.1144.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a newmethod for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
Schmitt, A., Nebreda, A.R., 2002. Inhibition of *Xenopus* oocyte meiotic maturation by catalytically inactive protein kinase A. Proc. Natl. Acad. Sci. U.S.A. 99, 4361–4366.
Thomas, P., Tubbs, C., Detweiler, C., Das, S., Ford, L., Breckenridge-Miller, D., 2005. Binding characteristics, hormonal regulation and identity of the sperm membrane progestin receptor in Atlantic croaker. Steroids 70, 427–433. https://doi.org/10.1016/j.steroids.2005.02.010.
Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., Tubbs, C., 2007. Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology 148, 705–718. https://doi.org/10.1210/en.2006-0974.
Thomas, P., 2004. Discovery of a new family of membrane progesterone receptors in vertebrates and detection of the alpha and beta subtypes in mouse brain, testis and uterus. Med.Chem. Res. 13, 202–209. https://doi.org/10.1007/s00044-004-0026-6.
Tischkau, S.A., Ramirez, V.D., 1993. A specific membrane binding protein for progesterone in rat brain: Sex differences and induction by estrogen. Proc. Natl. Acad. Sci. 90, 1285–1289. https://doi.org/10.1073/pnas.90.4.1285.
Tokumoto, M., Nagahama, Y., Thomas, P., Tokumoto, T., 2006. Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. Gen. Comp. Endocrinol. 145, 101–108. https://doi.org/10.1016/j.ygcen.2005.07.002.
Tokumoto, T., Tokumoto, M., Oshima, T., Shimizuguchi, K., Fukuda, T., Sugita, E., Suzuki, M., Sakae, Y.T., Akiyama, Y., Nakayama, R., Roy, S.R., Rahman, M.S., Pang, Y.F., Dong, J., Thomas, P., 2012. Characterization of multiple membrane progestin receptor (mPR) subtypes from the goldfish ovary and their roles in the induction of oocyte maturation. Gen. Comp. Endocrinol. 177, 168–176. https://doi.org/10.1016/j.ygcen.2012.03.005.
Tubbs, C., Pace, M., Thomas, P., 2010. Expression and gonadotropin regulation of membrane progestin receptor alpha in *Atlantic croaker*(*Micropogonias undulatus*) gonads: Role in gamete maturation. Gen. Comp. Endocrinol. 165, 144–154. https://doi.org/10.1016/j.ygcen.2009.06.017.
Wang, C., Liu, Y., Cao, J.M., 2014. G protein-coupled receptors: extranuclear mediators for the non-genomic actions of steroids. Int. J. Mol. Sci. 15, 15412–15425. https://doi.org/10.3390/ijms150915412.
Warrier, S.R., Tirumalai, R., Subramoniam, T., 2001. Occurrence of vertebrate steroids, estradiol 17 beta and progesterone in the reproducing females of the mud crab Scylla serrata. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 130, 283–294. https://doi.org/10.1016/S1095-6433(01)00385-3.
Wu, C.W., Dong, Z.Y., Chi, C.F., Ding, F., 2010. Reproductive and spawning habits of Sepiella maindroni of Zhejiang, China. Oceanol. Limnol. Sin. 41, 39–46.
Yano, I., 1987. Effect of 17α-hydroxy-progesterone on vitellogenin secretion in kuruma prawn, Penaeus japonicas. Aquactulture 61, 49–57. https://doi.org/10.1016/0044-8486(87)90337-1.
Zhu, Y., Bond, J., Thomas, P., 2003a. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc. Natl. Acad. Sci. U.S.A. 100, 2237–2242. https://doi.org/10.1073/pnas.0436133100.
Zhu, Y., Hanna, R.N., Schaaf, M.J.M., Spaink, H.P., Thomas, P., 2008. Candidates for membrane progestin receptors-Past approaches and future challenges. Comp. Biochem. Physiol. C. Tox. Pharm. 148, 38–389. https://doi.org/10.1016/j.cbpc.2008.05.019.
Zhu, Y., Rice, C.D., Pang, Y., Pace, M., Thomas, P., 2003b. Cloning, expression, and characterization of a membrane progestinreceptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. 100, 2231–2236. https://doi.org/10.1073/pnas.0336132100.