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Molecular cloning and expression of prostaglandin F2α receptor isoforms during ovulation in the ovarian follicles of *Xenopus laevis*

Zhiming Liu[a,b,*], Xiurong Su[a], Taiwu Li[a], Daodong Pan[a], Johnny Sena[b], Jasvinder Dhillon[b]

[a] *Faculty of Life Science and Biotechnology, Ningbo University, Ningbo, Zhejiang Province 315211, China*
[b] *Department of Biology, Eastern New Mexico University, Portales, NM 88130, USA*

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ABSTRACT

Prostaglandins F2α levels increase during ovulatory period in *Xenopus laevis* in response to stimulation by gonadotropins and progesterone. PGF2α exerts its effects on ovulation through interaction with its receptor (FP) in ovaries. Little is known about the characteristics of the FP receptor and its regulation during the ovulatory period in non-mammalian species. In the present study, two isoforms of prostaglandin F receptor (FP A and B) cDNAs were isolated from *Xenopus laevis* ovarian tissues using reverse transcription-polymerase chain reaction (RT-PCR) followed by rapid amplification of cDNA ends (RACE). The cDNAs of FP A and FP B were sequenced. In *Xenopus laevis* ovary, FP A and B mRNA levels were up-regulated during gonadotropin- and progresterone-induced ovulation *in vitro*. The mRNA level of FP B was higher than that of FP A. Moreover, FP A and FP B mRNA levels were measured in various tissues including eye, liver, lungs, heart, muscle, ovary, and skin. Overall, FP B mRNA level was approximately 10- to 100-fold higher than that of FP A, except in the muscle and skin where FP A mRNA level was comparable to that of FP B. The results suggest that in *Xenopus* ovarian follicles FP receptors play an important role during gonadotropin- and progesterone-induced ovulation.

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1. Introduction

Prostaglandins (PGs) mediate an array of physiological process such as inflammation, gastric secretions, blood flow; as wells as reproductive process such as ovulation, luteolysis, fertilization, implantation, and parturition [1–6]. Abnormalities in the production and function of PGs can lead to deficiencies in reproductive processes [2,7–9]. Five types of prostaglandins have been identified; PGD2, PGE, PGF2α, PGI2 and thromboxane (TXA). PGE and PGF2α along with their receptors (EP and FP) are the primary PGs involved in reproduction and are required for ovulation [10]. In fact, EP subtype 2 knockout mice fail to ovulate normally [11,12]. However, mice null for the FP receptor show no aberrations in ovulation [5]. In contrast, experimental evidence suggests that PGF2α is the primary PG involved in ovulation in sheep [13]. In several teleost fish species, PGs have been shown to increase during spontaneous or artificially induced ovulation, also indomethacin (PG biosynthesis inhibitor) blocks ovulation both *in vivo* and *in vitro* [14–20]. In *Rana esculenta*, PGF2α levels are higher during the reproductive period than in other periods [21]. Similarly, in *Rana pipiens,* ovulation is induced by treatment with exogenous PGF2α but not PGE [22]. In our previous experiments, we observed in *Xenopus leavis* (*X. laevis*) ovarian follicles, that PGF2α is synthesized during the periovulatory period similar to mammals [23]. Secondly, PGF2α synthesis is regulated by *de novo* transcription of COX-2 but not COX-1. We also found that *in vitro* inhibition of ovulation via actinomycin D could be rescued by treatment with exogenous PGF2α. In contrast, PGE2 did not rescue ovulation and in fact, increased inhibition. Together these studies suggest that PGs are important for ovulation in non-mammalian vertebrate as well, particularly PGF2α in the case of *X. laevis*. PG biosynthesis begins with the release of arahidonic acid (AA) from plasma membrane phospholipids via phospholipase A2 (PLA2) [24]. Free AA is then converted to PGs via cyclooxygenases (COX). PGs are then moved out of the cell by prostanoid transporters (PGT) where they bind to PG-specific, seven-transmembrane domain G-protein coupled receptors and elicit their actions in an autocrin–paracrine manner.

FP receptors (FP) have been cloned in a few species [25] and exist as two subtypes FP A and FP B [26]. Subtype FP B is a truncated form of FP A, differing in the carboxyl-terminal. Many studies have examined the role of PGF2α as well as the FP receptor during ovulation in mammals; however, little information regarding the involvement of the FP receptor in non-mammalian vertebrates is available. In fact, this is the first report of the isolation and molecular cloning of the FP receptor in a non-mammalian vertebrate species. The aim

* Corresponding author at: Department of Biology, Eastern New Mexico University, Portales, NM 88130, USA. Tel.: +1 575 562 2495; fax: +1 575 562 2192.
E-mail address: zhiming.liu@enmu.edu (Z. Liu).

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of our study was to isolate the *X. laevis* FP from ovarian follicles. FP cDNA was identified from *X. tropicalis* genome database using *in silico* screening. The putative *X. tropicalis* FP sequence was used as a reference for isolation of *X. laevis* FP A and FP B from the ovary. We also investigated the expression levels of FP A and B during gonadotropin-induced maturation and ovulation. Furthermore, we examined the expression levels of FP A and B in the eye, lung, liver, heart, leg muscle, ovary and skin of *X. laevis*.

2. Materials and methods

2.1. Animals and chemicals

Adult *X. laevis* females were purchased from Nasco (Fort Atkinson, WI). For all experiments, frogs were used within 5 days after arrival. Prior to tissue collection, frogs were deeply anesthetized with 0.2% tricaine methanesulfonate and spinally transected. Ovaries were surgically removed and used in the following experiments. Also, eye, heart, liver, lung, muscle, ovary, and skin were collected from each frog, stored in RNAlater RNA Stabilization Reagent (QIAGEN) and stored at −70° C until use in gene expression experiments. All procedures were reviewed and approved by the Animal Care and Use Committee of Eastern New Mexico University. General chemicals, human chorionic gonadotropin (hCG), antibiotics, progesterone (P4) and actinomycin D (ActD) were purchased from Sigma (St. Louis, MO).

2.2. Ovarian tissue culture

Ovarian tissues were surgically removed and placed in modified Barth's solution (MBS: 88 mM NaCl, 1 mM KCl, 0.33 mM Ca $(NO_3)_2{\cdot}4H_2O$, 0.41 mM $CaCl_2{\cdot}2H_2O$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.6) supplemented with streptomycin sulfate (50 mg/L) and penicillin (30 mg/L). Subsequently, ovarian tissues were cut into small pieces (approximately 100 mg of ovarian fragment containing full-grown ovarian follicles) and washed with MBS for 30 min under gentle agitation to remove the yolk components from damaged oocytes.

The effects of a general transcription inhibitor, actinomycin D (Act D), on maturation and ovulation were examined. In these experiments, progesterone P4 (maturation inducing hormone) or hCG (luteinizing hormone analog) was applied to induce oocyte maturation and ovulation. The series of incubations focused primarily on the inhibitory effects of Act D on maturation and ovulation. Ovarian fragments containing 30–50 full-grown (stage VI) oocytes were prepared and mass incubated with a low dose of hCG (5 IU/ml) for 30 min. The reason of this priming incubation is that our preliminary experiments showed that oocyte maturation rates in P4 treated samples are low, not infrequently and short-time hCG incubation seems to overcome this problem. Subsequently, fragments were washed three times with fresh MBS, then, incubated in 2 ml of MBS with P4 (5 μM) or hCG (25 IU/ml) in the presence or absence of Act D (5 μM). Incubation was carried out for 24 h. Ovarian fragments incubated in MBS only, were used as a negative control. After incubation, ovarian fragments were individually fixed with 2% trichloroacetic acid, and the number of germinal vesicle breakdown (GVBD) and ovulated eggs were counted under the light microscope. A second series of incubations were carried out as described above to examine FP mRNA expression during the ovulatory period. Ovarian fragments were washed three times with fresh MBS, then, incubated in 2 ml of MBS with P4 (5 μM) or hCG (25 IU/ml) in the presence or absence of Act D (5 μM) under gentle agitation. At time 0 (initial control), 2, 4, 6, 8, and 12 h, hCG and P4 treated tissue samples were collected serially for subsequent analysis. Act D treated samples were collected at the peak of ovulation (8 h) only. During the incubation period, conditions of ovarian fragments were inspected under the dissecting microscope to monitor maturational and ovulatory progression at the morphological level. FP cDNA levels in cultured ovarian tissues were quantified by reverse transcriptase real-time polymerase chain reaction (RT real-time PCR). Each individual experiment was conducted with ovarian tissue fragments from a single donor frog, and duplicate samples per treatment per incubation time were used. The experiment was repeated three times with a different donor frog.

2.3. Molecular cloning of X. laevis FP A and FP B cDNA

To isolate *X. laevis* (Xl) FP cDNA, genome sequences of *X. tropicalis*, a close relative of *X. laevis* were applied since its genome project has been ongoing and available (US Department of Energy Joint Genome Institute website: http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Firstly, a BLAST alignment search was conducted in the *X. tropicalis* genome by using amino acid sequence for human FP A as a query. As a result, putative *X. tropicalis* (Xt) FP was identified. Polymerase chain reaction (PCR) primer pairs, XtFP-Forward (5′-AAAGGGACTGGCTACGGTTT-3′) and XtFP-Reverse primers (5′-GTGTGTCTCGTGC ATTTGCT-3′) were designed based on putative XtFP nucleotide sequences. Next, total RNA was prepared from *X. laevis* ovarian tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. First-strand cDNA was synthesized from 5 μg of total RNA using oligo (dT) primer and PowerScript reverse transcriptase (BD Biosciences).

The PCR amplification was performed with the XtFP-Forward and XtFP-Reverse primers in a 50 μl final volume reaction mixture containing 1 μl of cDNA template, 5 μl of 10× reaction buffer, 200 μM of dNTPs, 1 μM of each primer, and 2.5 U of Platinum Taq DNA polymerase (Invitrogen). The PCR condition was 94 °C for 20 s, 54 °C for 30 s, and 72 °C for 1 min 10 s for 35 cycles with an initial 4 min 94 °C denaturation step and a final 7 min 72 °C extension step. After the initial PCR, products were analyzed on 1.5% agarose gel, however no bands were visible. Thus, the PCR product was diluted 100 times and used as a template in a second PCR (conditions were the same as above). Following the second PCR, products (expected size ∼500 bp) were analyzed on 1.5% agarose gel, T-A cloned with a pCR2.1 cloning vector (Invitrogen), and both strands of cDNA were sequenced using an automated sequencing 7 system with fluorescent dye terminator (PE Applied Biosystems, Foster City, CA).

Based on partial sequence information of the putative XlFP cDNA fragments, rapid amplification of cDNA ends (RACE) was performed to isolate the 5′- and 3′-ends of the cDNA using SMART RACE cDNA amplification kit (BD Biosciences, Clontech).

Briefly, the 5′- and 3′-RACE-Ready cDNA were synthesized from 5 mg of ovarian total RNA according to the manufacturer's protocol. For the 5′- and 3′-RACE of FP, the first PCRs were performed with Universal Primer A Mix (UPM; supplied with Kit) and gene specific primers (GSPs: 5′-RACE primer, 5′-GGCAAGGGACAGGATCCCCAAGAAGGAG-3′; 3′-RACE primer, 5′-CGGAAGCGTGATGGCAGTGGAACGATGC-3′) using 5′-or 3′-RACE-Ready cDNA as a template. PCR conditions were 94 °C for 3 min, then 5 cycles at 94 °C for 10 s and 72 °C for 3 min, 5 cycles at 94 °C for 10 s and 70 °C for 10 s and 72 °C for 3 min, 25 cycles at 94 °C for 10 s and 68 °C for 10 s and 72 °C for 3 min followed by a final extension at 72 °C for 5 min. Then, a second PCR (nested PCR) was performed using a 100- to 3-fold diluted first PCR mixture as a template with nested UPM and the following nested GSPs: for 5′-RACE, (5′-CCAAGTTCTGCTTGCCTGAATG-3′); and for 3′-RACE, (5′-CTGTCCCTTGCCATC TCTTTCT-3′). PCR conditions were 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 1.5 min for 30 cycles with a 3 min initial 94 °C denaturation step and a 5 min final extension step. Electrophoresis, subcloning, and