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Estrogen regulates the transcription of guppy isotocin receptors

Jianshuang Li, Likang Lyu, Haishen Wen, Yun Li, Xiaojie Wang, Yijia Yao, Xin Qi *

College of Fishery, Ocean University of China, Qingdao 266000, PR China

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ABSTRACT

Estrogen can regulate oxytocin receptor expression, which is mediated through estrogen receptors (ESRs) in mammals, initiating parturition. To further study the reproductive physiological process of ovoviviparous teleosts, guppies (*Poecilia reticulata*) were employed as the research model in the present study to identify the transcriptional regulation of ESRs on isotocin receptors (*itrs*). Since guppy embryos develop inside the ovary, in the present study, the levels of *itrs* in the ovarian stroma of pregnant female guppies treated with estradiol (E_2) *in vitro* were tested. E_2 increased only *itr2* mRNA levels 3 h post-treatment, with no variation in *itr1* mRNA expression levels. *In vivo*, pregnant guppies were immersed in different concentrations of E_2, significantly increasing the relative expression levels of *itr1* and *itr2* in the ovary. Moreover, based on dual-fluorescence *in situ* hybridization (ISH), both *esrs* and *itrs* mRNAs were localized in the same cells around the embryos in the ovary. To further investigate the regulation of *itr* transcription by estrogen, a luciferase reporter assay was performed, and the results demonstrated that E_2 treatment could induce E_2-dependent repression of luciferase activity in cells transfected with ESR1. However, overexpression of ESR2a or ESR2b caused a robust ligand-independent increase in *itr2* promoter activity. Deletion analysis of the *itr2* promoter indicated that there were novel potential ESR transcription factor-binding sites at −360 bp upstream of the 5′ end of the *itr2* promoter. Overall, our study provided novel results regarding the ESRs mediating the onset of parturition in ovoviviparous teleosts.

1. Introduction

In mammals, the oxytocin/oxytocin receptor (OT/OTR) system has been found to play a crucial role in a variety of physiological processes, including reproduction (Bethlehem et al., 2013), thermoregulation (Kasahara et al., 2013), food intake (Maejima et al., 2014; Blevins and Baskin, 2015) and social behavior (Ross and Young, 2009). The most established function of OT is to induce uterine contraction in parturition and milk ejection in lactation (Laycock and Meeran, 2012). As the homolog of OT in teleosts, isotocin (IT) is also involved in the regulation of reproduction. In the round goby (*Neogobius melanostomus*), the concentration of IT in the brain varies with the gonad development of male and female fish (Sokołowska et al., 2015). In the brain and ovary of catfish (*Heteropneustes fossilis*), the expression of *it* increased in the reproductive phases compared to the resting phase, especially in the spawning phase (Banerjee et al., 2015). IT can also stimulate the contraction of the tunica albuginea and oviduct to promote ovulation and spawning in gilthead seabream (*Sparus aurata L.*) (Piccinno et al., 2014) and Syngnathidae ovary (Scobell and Mackenzie, 2011). A study of guppies found that IT can induce premature parturition in females with shorter brood intervals (Venkatesh et al., 1992b). Isotocin receptor (ITR), a seven transmembrane domain receptor (Hausmann et al., 1995), mediates the function of IT or other nonapeptide hormones. Our previous study showed that Arg-vasotocin (AVT) promotes prostaglandin (PG) biosynthesis *via* ITR, which may induce delivery behavior in ovoviviparous guppies (Lyu et al., 2021). Overall, these findings indicate that the IT/ITR system plays an important role in ovulation and spawning in teleosts.

Steroid hormones are also involved in the development and maintenance of gonads (Chen et al., 2004). Estrogen, the most important steroid in females, shows a prominent effect, and the plasma concentration of estradiol (E_2) is positively correlated with the development of oocytes in many teleosts. In Pacific red snapper (*Lutjanus peru*), plasma E_2 concentration was significantly higher in late vitellogenesis and maturation of gonads, indicating E_2 plays an important role in oocyte final maturation and spawning activity (Dumas et al., 2018), which was also observed in other species (Prat et al., 1990; Pavlidis et al., 2000). The oocyte quality and fecundity of female fish can be affected by plasma E_2 concentration through regulating the transcription of vitellogenin (*vtg*) mRNA (Mushirobira et al., 2018). *In vivo* experiments on

* Corresponding author.
E-mail address: qx@ouc.edu.cn (X. Qi).

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pregnant guppies showed that E_2 induced premature parturition in females, indicating that E_2 plays an important role in the onset of parturition in ovoviviparous teleosts (Venkatesh et al., 1991). Moreover, a higher E_2 level in *Amphiprion ocellaris* female fish was related to parental behaviors, such as more active offspring rearing (DeAngelis et al., 2018). Accordingly, E_2 functions in regulating reproduction-related events, including oocyte development, mating behavior, ovulation and parturition.

In mammals, estrogen receptors (ESRs) mediate the effect of estrogen on OTR and have been well studied. A previous study showed that ESR1 was necessary for the induction of OTR binding in the brain by estrogen (Young et al., 1998). In rat uteri, the expression level of the *Otr* gene was upregulated by estrogen mediated by ESR1 and ESR2 (Murata et al., 2014), while only ESR1 but not ESR2 was confirmed as the main transcription factor of *otr* in ovine endometrium (Fleming et al., 2006). Recently, the transcriptional regulation mechanism between ESR and OTR has also been widely studied. In general, the actions of estrogen are mediated by several types of receptors, including nuclear receptors, such as ESR 1 and ESR 2, and putative membrane ESRs (Zuo and Wan, 2017). In addition to direct ESR/estrogen response element (ERE) interactions, several studies have reported that ESR may enhance gene expression by a nonclassical pathway by interacting with other transcription factors, such as specificity protein 1 (SP1) and activator protein-1 (AP-1) (Webb et al., 1999; Safe and Kim, 2004; Schanton et al., 2017; Barreto-Andrade et al., 2018). As a key regulator of *otr* expression, estrogen may be due to the SP1 transcription factor rather than the classical pathway through ESR/ERE to initiate OTR transcription (Kimura et al., 2003), and SP1 binds to a GC-rich sequence conserved in all mammalian cell types (Schanton et al., 2017). In summary, the ESRs mediating the estrogen effect on the transcription of OTR have been well established in mammals (Saville et al., 2000; Fleming et al., 2006). However, the regulatory mechanism between E_2 and ITR has not been determined in teleosts, especially in ovoviviparous teleosts with parturient behavior.

In our previous study, we confirmed that IT had a direct effect on cyclooxygenase 2 (*cox2*) expression, but no significant difference was observed in the induction of prostaglandin F_{2a} (PGF_{2a}) production, even though the PGF_{2a} concentration was higher than the control. Intraperitoneal administration of PGF_{2a} significantly induced premature parturition of guppies (Lyu et al., 2021). However, different results were also found in similar experiments that IT has no effect on inducing ovulation in the follicles *in vitro* of guppy (Venkatesh et al., 1992a). To determine whether the different effects of IT in inducing premature parturition are mediated by ESRs, in the present study, guppies were employed to explore the effect of E_2 on the expression of *itr* genes *in vivo* and *in vitro*, and the regulatory mechanism between *esrs* and *itrs* will be described. Our study provides novel information for further understanding the regulatory mechanism of E_2 on parturition mediated by *itr* in ovoviviparous teleosts.

2. Materials and methods

2.1. Animals

Female pregnant guppies were purchased from a fish farm in Anshan, Liaoning Province, China, and allowed to acclimatize for more than one week in tanks with a water temperature of 25 °C and a photoperiod cycle of 14 h light/10 h dark and fed pellet feed (Godzilla, China) without exogenous hormones twice a day. All animal experiments were conducted in accordance with the national guidelines and with the approval of the Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201).

2.2. Static incubation of ovarian stroma

Thirty-two female pregnant guppies were anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, 0.2 g/L). As described in a previous study (Lyu et al., 2021), with several modifications, the embryos were gently removed from ovarian tissues, and the rest were collected and washed with PBS three times. Then, the stroma of one female ovary was incubated in a well in a 24-well plate and starved for 2 h in 1 mL of medium 199 (without phenol-red) (MACGENE, China) supplemented with 100 units/mL penicillium and 100 μg/mL streptomycin (G-CLONE, China) in an incubator without CO_2 at 25 °C. After that, fresh culture medium containing 10% fetal bovine serum (FBS) and different concentrations of E_2 (10^{-5}, 10^{-6}, 10^{-7} M) or DMSO (0.01% (*v*/v)) was changed to the corresponding test well. Four biological replicates were prepared for each group. The stroma fragments of ovary were collected into TRIzol reagent (Invitrogen, USA) after incubation for 3 and 6 h at 25 °C and stored at −80 °C for subsequent RNA extraction and real-time PCR.

2.3. Effects of E_2 on itrs expression in ovarian stroma

Eighteen female pregnant guppies were acclimatized as described above and then immersed in 30 L of water containing 5 μg/L (1.8×10^{-8} M) or 100 μg/L (3.7×10^{-7} M) E_2 or DMSO alone for 3 days, as described in a previous study of E_2 treatment on guppies (Venkatesh et al., 1991). And the DMSO concentration in each group was 0.001% (v/v). Then, whole ovaries were removed from fish ($n = 6$ for each group), the embryos were peeled off, and the remaining ovarian stroma were collected and stored at −80 °C for real-time PCR to measure the expression of *itrs*.

2.4. RNA extraction, reverse transcription, and qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and purity of the total RNA were measured by UV spectrophotometry (OSTC, China), and agarose gel electrophoresis was used to detect RNA integrity. Then, one microgram of isolated RNA was reverse transcribed to complementary DNA by using a HiScript III RT SuperMix reagent kit (Vazyme, China) according to the manufacturer's protocols. qPCR was performed to detect the expression levels of *itr1* and *itr2* in guppy ovarian stroma. The primers for all genes are listed in Table 1.

The 10-μL qPCR mixture consisted of 1 μL of cDNA template, 0.2 μL of both primers, 5 μL of 2 × ChamQ SYBR Colour qPCR Master Mix (High ROX Premixed) (Vazyme, China), and 3.6 μL of RNAase-free water. The qPCR procedure was as follows: pre-denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 10 s and annealing temperature for 30 s, and a final extension at 72 °C for 30 s. The results from the melt curve analyses of the genes showed single peaks, confirming the specificity of the qPCR results. The efficiency of the qPCR is shown in Table 1. The *b-actin* RNA (*actb2*, GenBank accession number: NM_001297475.1) was used to normalize the relative expression of the genes (Shen et al., 2007), and the expression level of *actb2* gene does not vary between groups. The samples were run in triplicate. The relative expression levels of genes were analyzed by the $2^{-\Delta\Delta CT}$ method.

2.5. In situ hybridization (ISH)

Ovaries of pregnant female guppies were fixed with 4% paraformaldehyde (in 1 × PBS, pH 7.4), embedded in paraffin, and sliced into 7-μm slices. The sections were then deparaffinized, rehydrated, washed with PBS, and rinsed with 0.2 M HCl for 8 min before being digested with protease K (10 μg/mL) for 5 min at 37 °C. And then, the sections were washed with 0.1 M pH 8.0 triethanolamine-HCl with 0.25% acetic oxide for 10–15 min and 2 × SSC for 5 min. After incubated with hybrid buffer at 55 °C for 1 h, the sections were incubated with digoxigenin (DIG)-labeled sense or antisense probes at 55 °C overnight. After removing the nonspecific adsorption of probes by SSC, alkaline phosphatase-conjugated anti-DIG Fab fragments (1:400 dilution) was used to detect the DIG-labeled probes. And chromogenic development was conducted with an NBT/BCIP stock solution (Roche, Switzerland)

Table 1
Primers used for qPCR, dual-fluorescence *in situ* hybridization, promoter cloning and plasmid construction.

Name	Sequence(5′-3′)	
Primers for qPCR		Efficiency (%)
b-actin-F	GCCTATCTACGAGGGCTACGC	98.09
b-actin-R	TTGATGTCACGCACGATTTCC	
itr1-F	CCATCCACACCACCAAACAC	94.89
itr1-R	GCAAACATCCCGACAACC	
itr2-F	AGCGAAACGAAGAAGTAGCC	90.32
itr2-R	CGTTATGTCCCAGATGAGC	
Primers for preparation of dual-fluorescence ISH probes		
itr1-probe-F	CGCATTTAGGTGACACTATAGAAGCGCAGGTTGTCGGGATGTTTGC	
itr1-probe-R	CCGTAATACGACTCACTATAGGGAGACATCTTCGCCTTGGAGATGAGC	
itr2-probe-F	CGCATTTAGGTGACACTATAGAAGCGGGTGCTGCTACTCGCTCTGA	
itr2-probe-R	CCGTAATACGACTCACTATAGGGAGACAGCAGTCGTAAACGCCATTCC	
esr1-probe-F	CGCATTTAGGTGACACTATAGAAGCGTGCCCAGCGACCAATCAG	
esr1-probe-R	CCGTAATACGACTCACTATAGGGAGACAAGCACCTCCAGCCACGAA	
esr2a-probe-F	CGCATTTAGGTGACACTATAGAAGCGATAGACAAGAACCGACGCAAGA	
esr2a-probe-R	CCGTAATACGACTCACTATAGGGAGACATCAGTCCCATCATCAGCACCT	
esr2b-probe-F	CGCATTTAGGTGACACTATAGAAGCGCTCTGCACTGCACCCAACAT	
esr2b-probe-R	CCGTAATACGACTCACTATAGGGAGACACGACCCTTACCAAACTCCTG	
Primers for promoters cloning		
itr1 promoter F	CTTCAAAACAGGCGAGCACAT	
itr1 promoter R	TGCTGCAGCCTCGTCCTC	
itr2 promoter F	TGCATCCCAATATAACGGTTTAGT	
itr2 promoter R	CGTGCGCACCAGCGTCAG	
Primers for plasmids construction		
esr1-F	CTAGCGTTTAAACTTAAGCTTATGTATAAGAGGCAGAACCCGG	
esr1-R	CCACACTGGACTAGTGGATCCTCACAGGACCTGGGTGCAGT	
esr2a-F	CTAGCGTTTAAACTTAAGCTTATGGCCGCTGCCACCTCT	
esr2a-R	CCACACTGGACTAGTGGATCCTCAAGACGTGGCGGCGAT	
esr2b-F	CTAGCGTTTAAACTTAAGCTTATGGCGACTTCCCCTGGG	
esr2b-R	CCACACTGGACTAGTGGATCCTTAATTATCTTCCAAGGTGGTTTCC	
I1P-F	GCGTGCTAGCCCGGGCTCGAGCTTCAAAACAGGCGAGCACAT	
I1P-R	CAGTACCGGAATGCCAAGCTTTGCTGCAGCCTCGTCCTC	
I2P-F	GCGTGCTAGCCCGGGCTCGAGTGCATCCCAATATAACGGTTTAGT	
I2P-R	CAGTACCGGAATGCCAAGCTTCGTGCGCACCAGCGTCAG	
I2PP1-F	GCGTGCTAGCCCGGGCTCGAGCTGAGATTTAGCATTCATTATTAGGATCA	
I2PP2-F	GCGTGCTAGCCCGGGCTCGAGAGATCCAGCTGGACGCAGAG	
I2PP2-mut-R	CGTGCGCACCAGCGTCAGATTTAGTCAGCGATCACATCACACACAC	

according to the manufacturer's instructions. Sections were observed under microscope (Olympus, Japan).

2.6. Dual-fluorescence ISH of itrs and esrs mRNA

To confirm the direct regulation of E_2 on *itrs* expression, dual-fluorescence ISH was performed as described previously (Lyu et al., 2021), with modifications. First, ovaries of pregnant female guppies were fixed with 4% paraformaldehyde (in 1 × PBS, pH 7.4), embedded in paraffin, and sliced into 7-μm slices. After deparaffinized, rehydrated, washed with PBS, sections were incubated with 3% H_2O_2 for 20 min and protease K for 5 min at 37 °C. Then, the sections were washed with 0.1 M pH 8.0 triethanolamine-HCl with 0.25% acetic oxide for 10–15 min, 2 × SSC for 5 min, and incubated with hybrid buffer at 55 °C for 1 h. A mixture of DIG-labeled *itr* probe and biotin-labeled *esr* probe was used for dual-fluorescence ISH, and related primers are listed in Table 1. After hybridization, the sections were washed with SSC, blocked with blocking buffer, and then incubated for 1 h with HRP-conjugated anti-DIG antibody (diluted 1:400 with blocking buffer, Roche, Switzerland). After rinsing with PBS, the sections were incubated with substrates labeled with Alexa Fluor 594 (Invitrogen, USA) until the staining was ready for observation. Subsequently, to remove interference from endogenous biotin, the sections were washed and incubated with 3% H_2O_2 and endogenous biotin blocking buffer. Then, HRP-conjugated streptavidin secondary antibody (Proteintech, USA) was used to incubate sections for 1 h, and sections were incubated with substrates labeled with Alexa Fluor 488 (Invitrogen, USA) until observation. Finally, DAPI was used to detect the nucleus of the cell before the appropriate visible signals were observed under a fluorescence microscope (Echo, America).

2.7. Promoter sequence analysis

The genomic DNA of guppies was isolated by the Genomic DNA Extraction Kit (Tiangen, China) according to the manufacturer's instructions. The locations of guppy *itr* genes were obtained from the GenBank of the National Center for Biotechnology Information (NCBI), and then, the 2000 bp putative promoter sequences were searched in the genome by TBtools (Chen et al., 2020). After amplification and purification with the primers (Table 1), the promoter sequences were subcloned into the pCE2-TA/Blunt Zero vector (Vazyme, China) and verified by sequencing at the Beijing Genomics Institution (BGI). In addition, Jaspar (http://jaspar.genereg.net/), an online algorithm, was used to predict the potential transcription factor-binding sites of *itr* promotor sequences.

2.8. Dual luciferase reporter assay

Construction of recombinant plasmids for reporter gene assays was performed by a ClonExpress® Ultra One Step Cloning Kit (Vazyme, China) according to the manufacturer's instructions. The primers used for plasmid construction are described in Table 1, and all of the recombinant plasmids were verified by sequencing. Different lengths of *itr* promoter sequences were cloned into the pGL3-basic plasmid, and full-length guppy *esr1* (GenBank accession number XM_008397690.2), *esr2a* (XM_017302316.1), and *esr2b* (XM_008398650.2) were cloned into the pcDNA 3.1 plasmid. To examine the promoter activities in response to E_2 in HEK 293 T cells, cells were seeded in 24-well plates at a density of 1 × 10^5 cells/mL in phenol-red free Dulbecco's modified Eagle's medium (G-CLONE, China) supplemented with 10% FBS. Xfect™ Polymer (Takara, Japan) was used to transfect cells with 500 ng of the *itr* promoter

reporter plasmid, 300 ng of the *esr* expressing plasmid, and 50 ng of pRL-TK (to normalize transfection efficiency) containing the *Renilla* luciferase sequence. After transfection for 12 h at 37 °C, the cells were treated with a 10^{-7} M final concentration of E_2 or DMSO alone (0.01% (*v*/v)) (Pak et al., 2006; Suzuki et al., 2010). After incubation for 36 h, the cells were harvested to measure luciferase activities by a Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's directions. Each experiment was repeated three times.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 19.0. All data are presented as the mean ± SEM. The data were tested for normal distribution and homogeneity of variance. To identify significant differences between more than two groups, we used one-way ANOVA followed by Tukey's *post hoc* analysis. Student's *t-test* was used to detect significant differences between the two groups. All significance was defined as $P < 0.05$.

3. Results

3.1. Effects of E_2 on the expression of itrs in pregnant guppy stroma of ovary in vivo

To determine whether E_2 regulates *itrs* expression, 18 pregnant guppies were exposed to water containing different concentrations of E_2, and the stroma of the ovary was collected to measure the gene expression levels. The results showed that the expression levels of both *itr1* and *itr2* in pregnant guppy stroma of ovary were upregulated significantly under 5 μg/L (1.8×10^{-8} M) of E_2 ($P < 0.05$) compared with the control group (Fig. 1).

3.2. Effects of E_2 on the expression of itrs in pregnant guppy stroma of ovary in vitro

To further explore the effect of E_2 on *itrs* expression, *in vitro* static incubation of ovarian stroma was performed. As shown in Fig. 2, the expression level of *itr2* was increased significantly ($P < 0.05$) after 3 h of incubation under 10^{-7} M of E_2, and it was maintained at a high level in the other two treatment groups (Fig. 2B). However, no significant difference in *itr1* expression levels was observed between the treatment and control groups after 3 h of incubation (Fig. 2A), and decreased expression appeared after 6 h of incubation under 10^{-7} M of E_2 compared with the control group (Fig. 2C).

3.3. Colocalization of itrs and esrs in pregnant guppy stroma of ovary

To determine the regulatory function of ESRs on *itrs*, dual-fluorescence *in situ* hybridization of 2 *itrs* and 3 *esrs* (*esr1*, *esr2a*, *esr2b*) was performed. As shown in Fig. 3 and Fig. 4, embryos that developed in the ovary were enveloped by a monolayer of follicular cells. Positive signals of *itrs* and *esrs* were colocalized in the follicular cell layer, suggesting that a potential transcriptional regulatory relationship between *itrs* and *esrs*. The ISH results of sense and antisense probe were compared in Supplementary Fig. 1, and the gene expression locations have been labeled.

3.4. Sequence analysis of itr promoters

To characterize the regulatory mechanism of E_2 on the *itrs* expression in guppies, a dual luciferase reporter assay was performed. A total of 1810 bp and 1646 bp sequences from the upstream regions of guppies *itr1* and *itr2*, respectively, was isolated by PCR. Promoter analysis results (Fig. 5) showed that the guppy *itr* promoter region contained multiple putative binding sites for the transcription factor SP1, which mediates the transcriptional regulation of steroid hormone receptors. Putative SP1 sites were found at positions −53 to −62 (CCCCTCTCTT), −512 to −516 (TGCCC) and − 585 to −594 (GTGGCGGTGA) in the *itr1* promotor region and −23 to −27 (GCACA), −377 to −381 (GCACA), −383 to −391 (GTGGTGGGA), −396 to −400 (GCACA), −449 to −453 (GCACA), −1015 to −1019 (GCACA), −1080 to −1084 (GTGCA), −1148 to −1157 (GGGGATCGGT) and −1295 to −1299 (GGGCA) in the *itr2* promotor region relative to the translational start site (ATG + 1). There were also putative AR sites at −1707 to −1721 (AGGAACACGTAGCCG) and −108 to −122 (GAGAACAGAGTAACC) in the *itr1* and *itr2* promoter regions, respectively. ERE half sites were found at −46 to −50 (TGACC), −116 to −120 (TGACC), −1307 to −1311 (GGTCA), −1554 to −1558 (TGACC) and at −496 to −500 (GGTCA), −1108 to −1112 (GGTCA), −1120 to −1124 (GGTCA) in the *itr1* and *itr2* promoter regions, respectively. No complete EREs were found by computer-assisted analysis of the *itrs* upstream regions of the guppy.

3.5. Dual luciferase reporter assay

Previous results have shown that *itrs* are co-expressed with all 3 types of the *esrs* in the ovary. Therefore, luciferase reporter assays were performed to test the effects of E_2 on *itrs* transcription in HEK 293 T cell line. As shown in Fig. 6, compared with the control group, the cells co-transfected with all 3 *esr* expression vectors and *itr2* promoter reporter

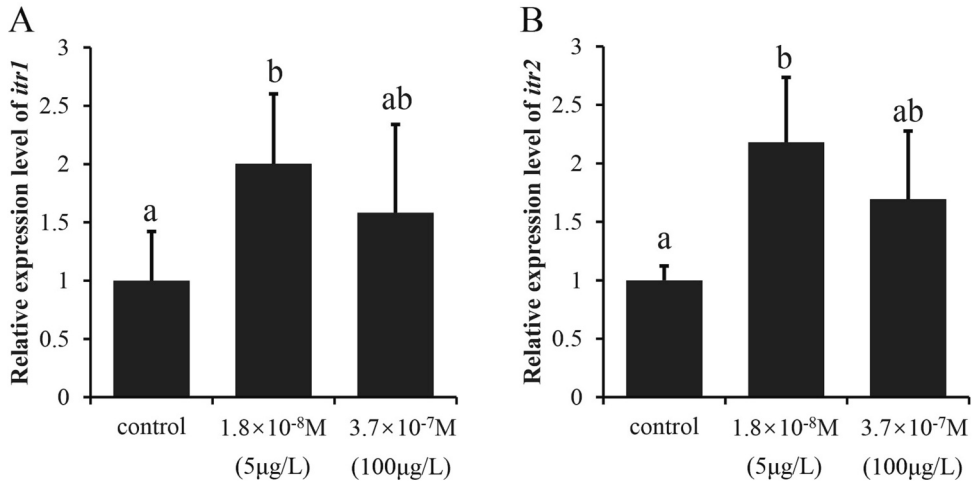

Fig. 1. Effect of E_2 on *itrs* expression in the stroma of the ovaries of pregnant guppies ($n = 6$). (A) The expression level of *itr1*. (B) The expression level of *itr2*. Significant differences are shown by different letters in each group ($P < 0.05$).

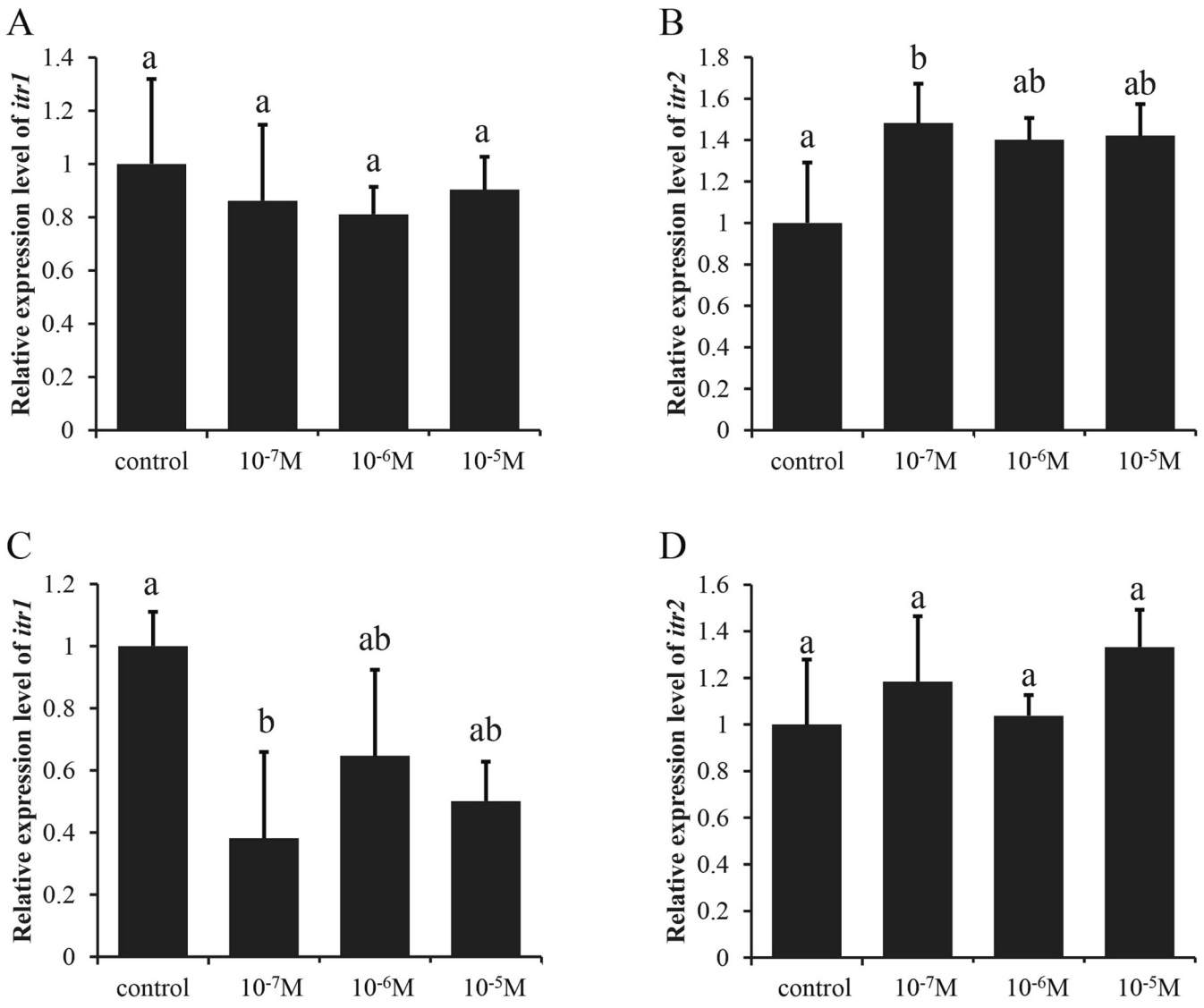

Fig. 2. Effect of E_2 treatment on the expression of *itrs* in ovarian stroma ($n = 4$). (A) The relative expression level of *itr1* after 3 h of treatment. (B) The relative expression level of *itr2* after 3 h of treatment. (C) The relative expression level of *itr1* after 6 h of treatment. (D) The relative expression level of *itr2* after 6 h of treatment. Significant differences are shown by different letters ($P < 0.05$).

vectors had a significant increase in the relative activity of luciferase ($P < 0.05$), while that was not observed in *itr1* promotor reporter vectors. Therefore, co-transfection of *esrs* was more effective in inducing *itr2* promoter activity, and ESR transcription factor-binding sites existed in the promoter region of *itr2*.

To further investigate the ESR-dependent E_2-responsive regions in the guppy *itr2* promoter, deletion mutants of the *itr2* promoter sequence were constructed *via* PCR, and then the reporter vectors of the corresponding mutants were transfected into HEK 293 T cells for luciferase assays. The results (Fig. 7) showed that E_2 treatment could induce E_2-dependent repression of luciferase activity in cells transfected with ESR1. However, overexpression of ESR2a or ESR2b caused a robust ligand-independent increase in *itr2* promoter activity. The relative luciferase activity decreased as the *itr2* promoter sequence was deleted to −769 bp upstream of the 5′ end. However, when the sequence was deleted to −360 bp, the promoter activity increased significantly. To further determine the site on the *itr2* promoter that is required for the ligand-independent and ligand-dependent actions of ESR1, ESR2a and ESR2b, the mutant construct with a deleted putative SP1 site was subjected to a luciferase assay. The results showed that there was no significant effect of the deletion on the basal activity of the −360 bp promoter construct, thus, this site was not critical for ESR-induced *itr2* promoter activity. Therefore, there were novel ESR transcription factor-binding sites in −360 bp upstream of the 5′ end of the gene.

4. Discussion

Steroid hormones are essential for the maintenance of pregnancy and successful parturition in mammals, including estrogen, androgen, progesterone, mineralocorticoid and glucocorticoids, among which, estrogen is known for its role in female reproductive physiology. It has been found that estrogen can affect placental function, fetal growth and development, parturition initiation, neuropeptide and glycoprotein release and other physiological processes in humans and primates (Chardonnens et al., 1999; Bukovsky et al., 2003). In previous studies, E_2 was shown to induce the expression of OTR through interaction with its receptor. For example, in human placental villus cancer cells, E_2 can upregulate the mRNA and protein levels of OTR through estrogen receptors, mainly through ESR1 (Kim et al., 2017a), and ESR1 may be activated by estrogen in ovarian follicles or growth factors in stroma and then regulate gene expression in ovine endometrium (Fleming et al., 2006). Additionally, E_2 induces the expression of *Otr* and increases the

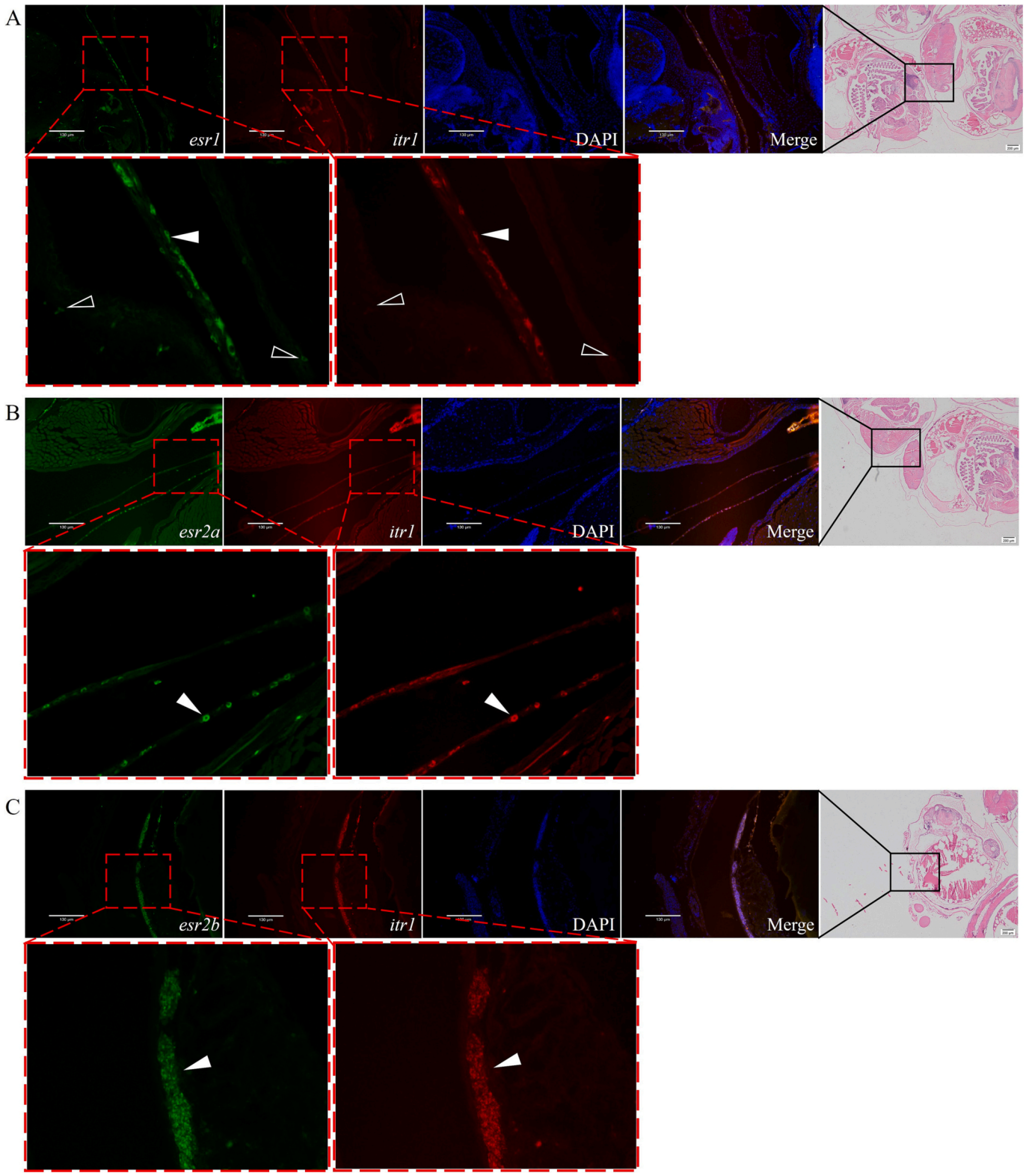

Fig. 3. Dual-fluorescence *in situ* hybridization of *itr1* and *esrs* in ovaries of pregnant guppies. The red regions represent *itr1* (stained with Alexa Fluor 594), the green regions represent *esrs* (stained with Alexa Fluor 488), and the blue regions represent cell nucleus (stained with DAPI). The white arrowheads indicate the co-expression region of *itr1* and *esrs*. The open arrowheads represent a single signal of the gene. Scale bar, 130 μm. H&E staining of the ovaries to show histological morphology. Scale bar, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

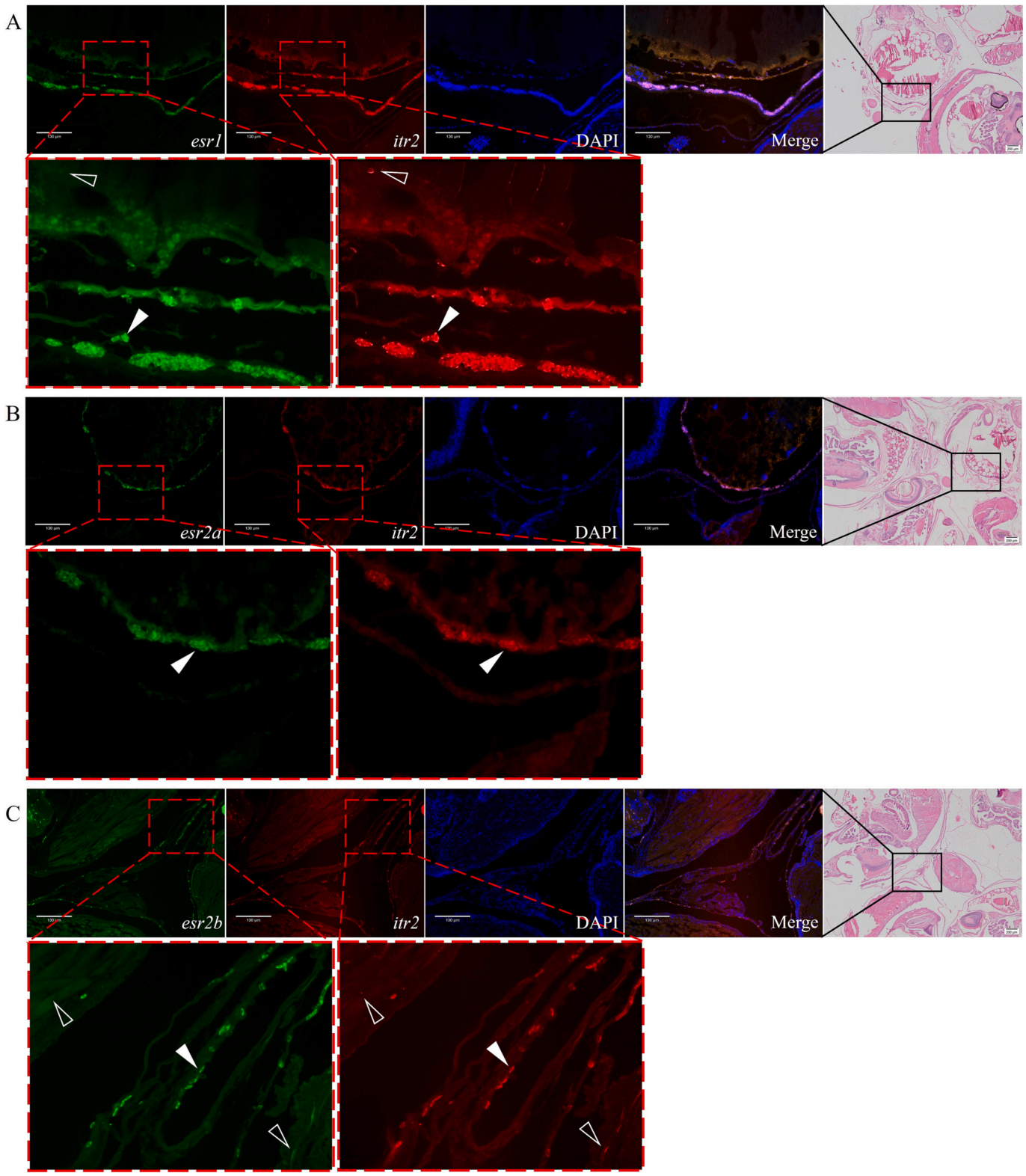

Fig. 4. Dual-fluorescence *in situ* hybridization of *itr2* and *esrs* in ovaries of pregnant guppies. The red regions represent *itr2* (stained with Alexa Fluor 594), the green regions represent *esrs* (stained with Alexa Fluor 488), and the blue regions represent cell nucleus (stained with DAPI). The white arrowheads indicate the co-expression region of *itr2* and *esrs*. The open arrowheads represent a single signal of the gene. Scale bar, 130 μm. H&E staining of the ovaries to show histological morphology. Scale bar, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

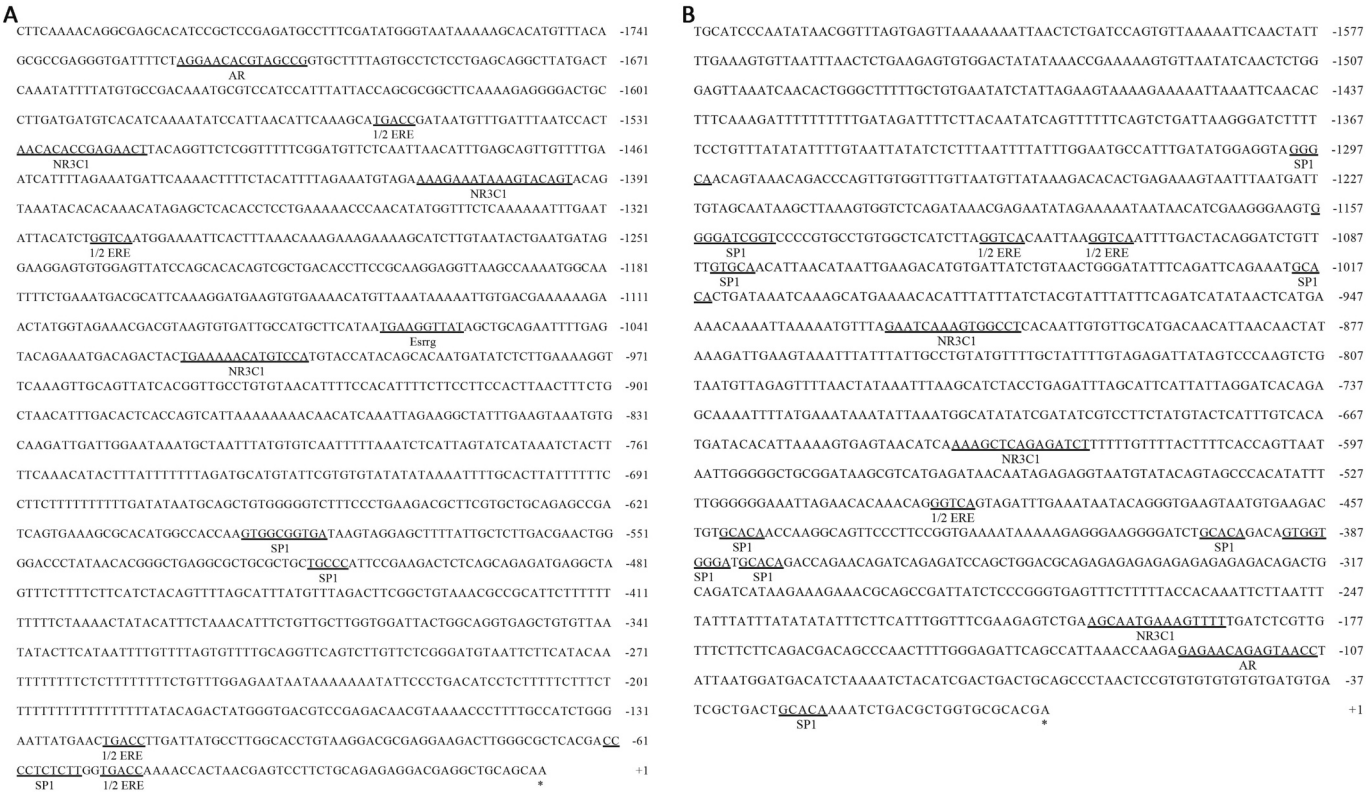

Fig. 5. Sequence analysis of *itr* gene promoters. The transcription start site is noted by '*' and designed as position +1. The transcription factor-binding sites predicted by Jaspar are marked in the figs. (A) The promoter sequence of *itr1*. (B) The promoter sequence of *itr2*.

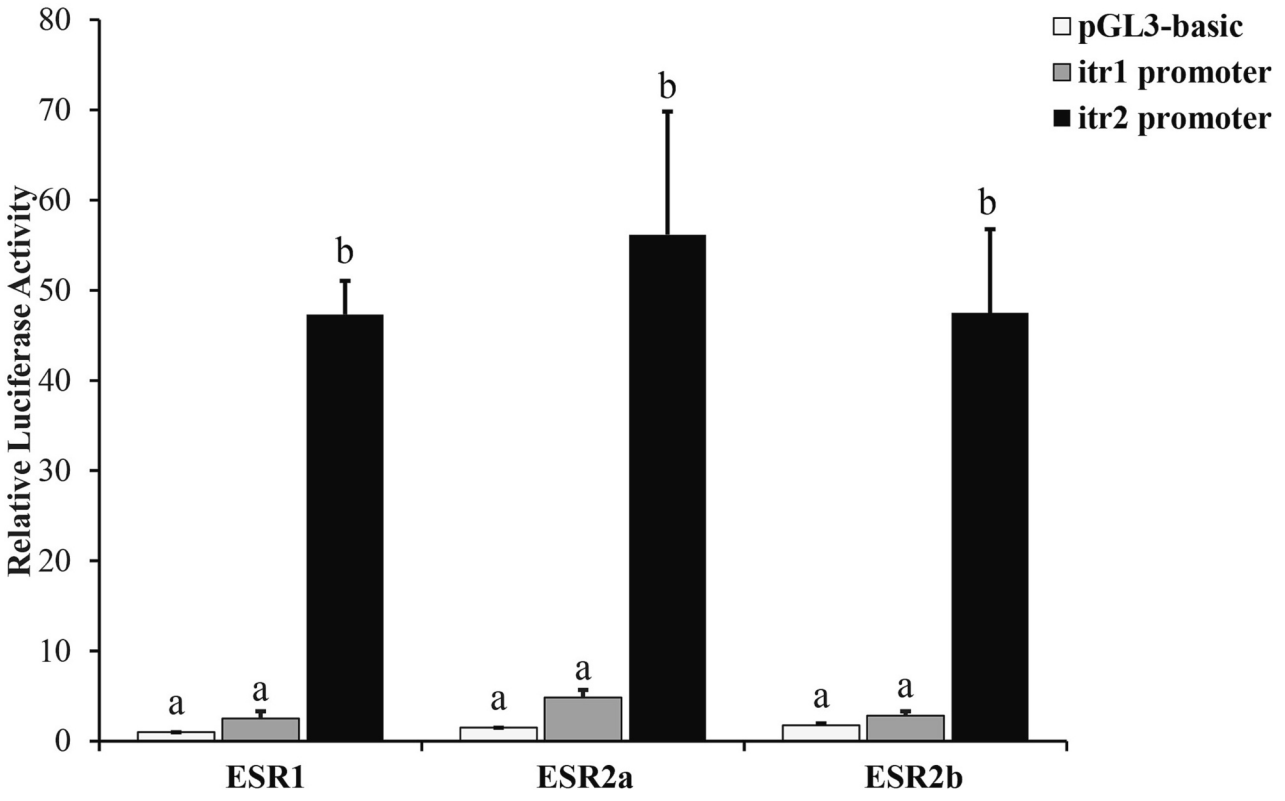

Fig. 6. The effect of E_2 on the *itr1* and *itr2* gene promoter activities ($n = 3$). The empty pGL3-basic vector was used as an internal reference, and significant differences are shown by different letters ($P < 0.05$).

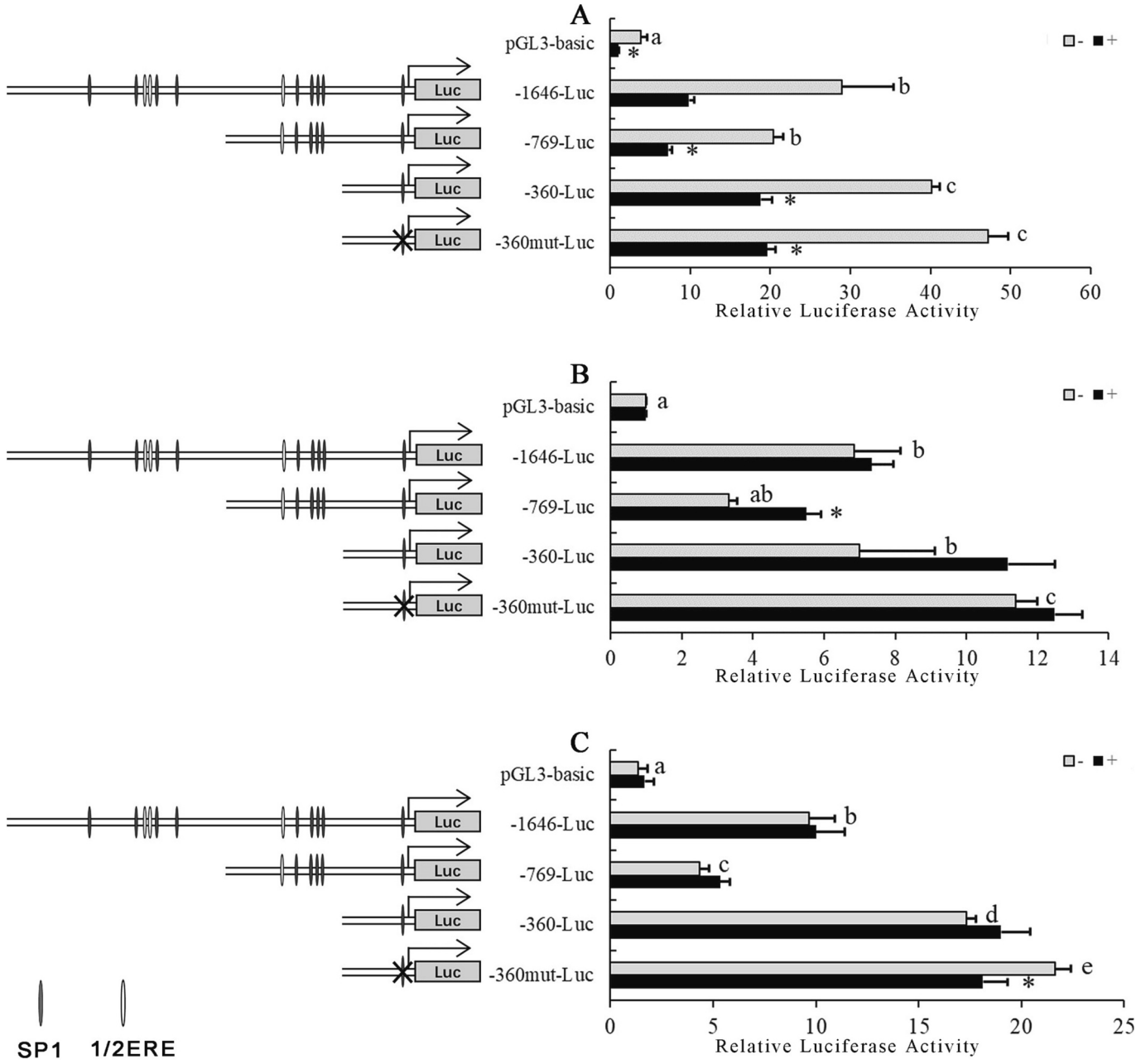

Fig. 7. Deletion analysis of guppy *itr2* gene promoter. Left panel: schematic representation of the 5′-deletion constructs of the *itr2* promoter and the putative transcription factor-binding sites. Right panel: the relative luciferase activities determined by transfecting different promoter fragments of *itr2* with the results normalized with the empty pGL3-basic vector. A, B and C represent co-transfection with ESR1, ESR2a, and ESR2b expression vectors, respectively. After transfection, the medium was changed with 10^{-7} M E_2 or vehicle for 36 h, and luciferase activities were measured (n = 3). Significant differences between different groups are noted by different letters ($P < 0.05$), and '*' represents significant differences between E_2 and vehicle treatment.

protein level of OTR synthesized in the uterus in ovariectomized rats (Fuchs et al., 1983; Soloff et al., 1983; Larcher et al., 1995). Moreover, OT/OTR plays an important role in various physiological processes of mammals, and its initial recognized function is to stimulate uterine smooth muscle contraction and promote parturition (Kim et al., 2017b). Therefore, E_2 can affect parturition by regulating the expression of OTR. In the present study, the effect of E_2 on isotocin receptor expression was also observed in the ovarian stroma of pregnant female guppies by both *in vivo* and *in vitro* experiments. The results showed that E_2 enhanced the expression of both *itrs*, especially *itr2*, which may be because *itr2* is more conserved in most teleosts and may have the same function as OTR, while *itr1* may have some defined functions (Yang et al., 2018). In addition, the results of dual-fluorescence ISH showed that *esrs* and *itrs* were co-localized in the cells around the embryos, indicating a regulatory relationship between the two genes. Similar results were found in the human placenta syncytium, in which ESR and OTR proteins were also co-localized, and both ESR proteins were located in the nucleus of trophoblast cells (Albrecht et al., 2006; Kim et al., 2017a). Therefore, ovoviviparous teleosts may promote the parturition process in late gestation through the interaction between the ESR and ITR. In the present study, the relative luciferase activity of cells containing the *itr2* promoter reporter plasmid was significantly enhanced compared with that of the controls and significantly higher than that of *itr1*. These data were consistent with the results of the *in vitro* experiment showing that E_2 mainly induced *itr2* expression in the ovarian stroma of guppies.

The functions of estrogen are mediated by estrogen receptors,

including nuclear receptors (NRs) and membrane receptors (GPR30) (Mårtensson et al., 2009). ESRs can act by 1) directly binding to the ERE in the promoter sequence of target genes (Saville et al., 2000), 2) regulating other transcription factors (including SP1, AP1, *etc.*) by protein-protein interactions to indirectly regulate the transcription of target genes (Safe and Kim, 2008), and 3) activating extranuclear signaling pathways to regulate target genes, such as nitric oxide production and MAPK, calcium mobilization or AKT pathways (Burns et al., 2011). In mammals, estrogen is considered a key factor regulating OTR expression, however, the promoter sequence of all OTR studied lack intact ERE, suggesting that the transcriptional regulation between estrogen and OTR may be achieved through 1/2 ERE or indirect regulation, instead of the direct interaction of ESR/ERE (Kimura et al., 2003). In the promoter regions of guppy *itr* genes, no complete ERE palindromic sequences (5'-GGTCAnnnTGACC-3') (Klein-Hitpass et al., 1988) were found, while several 1/2 ERE and SP1 transcription factors-binding sites were predicted in the sequences. 1/2 ERE, containing only one side of the ERE sequence, has the ability to bind to ESR and cooperate with the interaction of ESR/SP1 when the SP1 sequence appears near its sequence (Klinge, 2001). In zebrafish, there were four 1/2 ERE on the promoter sequence of *vtgAo1*, and the reporter gene could be activated in the presence of ESR1 and E_2 (Muncke, 2006). Moreover, ESR1 can actively modulate estrogen-dependent *vtg* gene expression through the typical ERE binding site on the *vtg* promoter (Mushirobira et al., 2018). However, in the present study, ESR1 mediated E_2-dependent repression of *itr2* promoter activity in cells, which was also observed in HepG2-ERa cells, in which E_2 repressed PROS1 transcription depending upon the interaction of ESR1-SP1 (Suzuki et al., 2010). SP1 exists in all mammalian cell types and is involved in the expression regulation of tissue specific, cell cycle, and signaling pathway response genes (O'Connor et al., 2016). Several studies have shown that ESR can mediate the estrogen response of various proteins by interacting with SP1. The binding efficiency between SP1 and ESR was affected by the relative expression levels of ESR1 and ESR2, which further influenced the transcription of target genes. Activation of ESR1 in adipocytes increased the nuclear content of SP1 protein, the interaction of SP1/ESR1, and the binding efficiency of SP1 to the *Slc2a4* gene promoter, eventually leading to the increased expression of *Slc2a4* (Barreto-Andrade et al., 2018). In contrast to the estrogen-dependent effects of ESR1, ESR2a and its splice variants increased GnRH or Arginine vasopressin (AVP) promoter activity in a ligand-independent fashion (Pak et al., 2006; Pak et al., 2007), which was consistent with our results that overexpression of ESR2a or ESR2b caused a robust ligand-independent increase in *itr2* promoter activity. Collectively, these data suggest that ESR1 and ESR2 may have different physiological functions or regulatory mechanisms of gene transcription.

Although the SP1 binding site is a necessary *cis*-acting element for the basic activity of the ovine *otr* promoter, which can mediate the transcriptional regulation of ligand-activated ESR or PR on OTR (Fleming et al., 2006), the deletion of the SP1 binding site at −23 bp showed no significant effect on the basal activity of the mutation construct, and this GC-rich region was not critical for ESR-mediated *itr2* promotor activity. In addition, the results of sequence deletion of the *itr2* promoter showed that the luciferase activity of the total length promoter was significantly lower than that of other deleted sequences, which was also observed in *otr* promoter deletion experiments in ovine (Kimura et al., 2003). Accordingly, the expression of the guppy *itr2* gene was inhibited by transcription factor-binding site between −769 bp and −360 bp of the promoter, while the suppression was removed when this region was deleted in the construct, leading to a robust increase in promoter activity. Therefore, there could be novel ESR transcription factor-binding sites between the 5′ end and − 360 bp in the promoter region of the *itr2* gene, which initiates gene transcription.

5. Conclusion

The present study is the first to explore the transcriptional regulation relationship between ESR and ITR in ovoviviparous teleosts. We found that E_2 can induce *itr* expression in the ovarian stroma of pregnant female guppies both *in vivo* and *in vitro*. Cells that expressed both *esr* and *itr* mRNA were localized in ovary tissues around the developing embryos. The results of the luciferase assay clearly showed that there were potential transcriptional factor-binding sites in the *itr2* promoter sequence, which may mediate the regulation of ESR on *itr2* expression. Based on the regulatory mechanism of mammalian parturition, the parturition process of ovoviviparous teleosts may be initiated through the regulation of ESR on *itr* expression. These results provide data supporting the interaction between ESR and ITR and provide novel insights into the mechanism of parturition initiation in ovoviviparous teleosts.

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Ethical approval

All procedures involved in dealing of fish in this study were approved by Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201) prior to the initiate of the study. The studies did not involve endangered or protected species. And all experiments were performed in accordance with the guidelines of National Research Council for the Care and Use of Laboratory Animals.

CRediT authorship contribution statement

Jianshuang Li: Formal analysis, Investigation, Writing – original draft, Visualization. **Likang Lyu:** Writing – review & editing. **Haishen Wen:** Supervision. **Yun Li:** Investigation. **Xiaojie Wang:** Writing – review & editing. **Yijia Yao:** Investigation. **Xin Qi:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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