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Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*

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Abstract

Japanese flounder (*Paralichthys olivaceus*), a teleost fish that has XX (female)/XY (male) sex determination system, exhibits temperature-dependent sex determination. We have previously shown that high water temperature or an aromatase inhibitor treatment causes the sex-reversal from genetic females to phenotypic males and suppression of mRNA expression of *ovary-type P450 aromatase* (*cyp19a1*), a steroidogenic enzyme responsible for the conversion of androgens to estrogens, in Japanese flounder. In the present study, we demonstrate that high water temperature treatment suppresses specifically mRNA expression of the forkhead transcription factor gene *foxl2*, and *follicle-stimulating hormone receptor* (*fshr*) in gonads during early sex differentiation. Moreover, transient transfection assay shows that the flounder Foxl2 and cAMP analog can activate the *cyp19a1* gene transcription *in vitro*. These results strongly suggest that FSH signaling and Foxl2 are involved in the transcriptional regulation of *cyp19a1* gene during gonadal sex differentiation in Japanese flounder with temperature-dependent sex determination.

Keywords: P450 aromatase; cyp19; foxl2; Follicle-stimulating hormone receptor; Temperature-dependent sex determination; Japanese flounder

Cytochrome P450 aromatase, the product of *cyp19* gene, is the steroidogenic enzyme responsible for conversion of androgens to estrogens. In vertebrates, this enzyme is expressed in various tissues and plays an important physiological role by regulating estrogen biosynthesis. Recently, it has been reported in non-mammalian vertebrates that expression of *aromatase* mRNA in the ovary is extremely higher than that in the testis during gonadal sex differentiation period [1–4]. Moreover, an aromatase inhibitor (fadrozole) has been shown to induce sex-reversal of genetic females to phenotypic males in chicken [5], reptiles [6–8], amphibians [9], and teleost fishes [10,11]. These findings indicate that aromatase plays pivotal roles in ovarian differentiation in non-mammalian vertebrates.

Japanese flounder (*Paralichthys olivaceus*) is a teleost fish which has XX (female)/XY (male) sex determination system [12]. The genetic females can be completely sex-reversed to phenotypic males when the larvae are reared at a high water temperature (27 °C) during the sex differentiation period [4]. Therefore, the flounder provides an excellent model to study molecular mechanism of temperature-dependent sex determination (TSD). Previously, we demonstrated that a high water temperature, fadrozole, or tamoxifen (anti-estrogen) treatment caused sex-reversal from genetic females to phenotypic males and suppression of *ovary-type aromatase* (*cyp19a1*) mRNA expression in gonads [4,11,13]. These results strongly suggest that suppression of *cyp19a1* mRNA expression and the resultant inhibition of estrogen biosynthesis trigger the sex-reversal of the genetic females by high water temperature.

To elucidate the mechanism of transcriptional regulation of *cyp19a1* gene in Japanese flounder with TSD, we

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first isolated 5′-flanking regions of *cyp19a1* gene from Japanese flounder. Then, we cloned the flounder homologs of the forkhead transcription factor gene *foxl2*, *follicle-stimulating hormone receptor* (*fshr*), and *luteinizing hormone receptor* (*lhr*), which are known to be implicated in ovarian development in vertebrates, and investigated whether they are involved in the transcriptional regulation of the *cyp19a1* gene in Japanese flounder.

Materials and methods

Animals. All genetically female broods of Japanese flounder were produced artificially by mating gynogenetic females with sex-reversed, gynogenetic males as described previously [4]. Larvae were kept at 18 °C up to 29 days after hatching (dah). Phenotypic females and males were produced by rearing the genetically female broods at 18 °C and 27 °C, respectively, from 30 to 100 dah as described previously [4].

Isolation and sequence analysis of 5′-flanking region of Japanese flounder cyp19a1 gene. The 5′-flanking region of Japanese flounder cyp19a1 gene was isolated from the flounder genomic DNA by PCR with the *cyp19a1*-specific primers (first PCR, 5′-AACAACCTGCACAAGACTGC-3′; nested PCR, 5′-ACTGCACAAACTGGGCGACC-3′) using Universal GenomeWalker Kit (Clontech Laboratories Inc., Palo Alto, CA) as described previously [14]. PCR products were electrophoresed on a 2% agarose gel, purified, inserted into the pT7Blue T-vector (Novagen, Madison, WI), and sequenced by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA) using ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems).

Isolation and sequence analysis of Japanese flounder foxl2, fshr, and lhr cDNAs. One microgram total RNA extracted from the adult ovaries using ISOGEN (Nippongene, Tokyo, Japan) was reverse-transcribed using RNA PCR Kit (Applied Biosystems) at 42 °C for 30 min. To isolate Japanese flounder *foxl2* cDNA, PCR was performed with the degenerate primers (5′-GAGAAGMGBCTYACGCTGTCCGG-3′ and 5′-CCCART AWGAGCARTGCATCAT-3′) using the ovarian cDNA as a template in the PCR mixture [0.2 mM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 1 unit Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems)]. PCR conditions were as follows: preheating at 95 °C for 10 min, 30 cycles of PCR at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were subcloned and sequenced according to the methods described above. To determine the 5′-end and 3′-end sequences of the flounder *foxl2* cDNA, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was performed with the *foxl2*-specific primers using GeneRacer Kit (Invitrogen, Carlsbad, CA) as described previously [14]. PCR products were subcloned and sequenced according to the methods described above.

To isolate Japanese flounder *fshr* and *lhr* cDNAs, PCR was performed with the degenerate primers (5′-CCNRNVSCNGAYGMBTTYAAYCCB TGYGARGA-3′ and 5′-GCNGANABRGCRWARAANGARATVGG NGCCATRCA-3′), which were designed in the conserved transmembrane domains, using the ovarian cDNA as a template in the PCR mixture. PCR conditions were as follows: preheating at 98 °C for 3 min, 40 cycles of PCR at 98 °C for 30 s, 50 °C for 1 min, 68 °C for 15 min, and a final extension at 68 °C for 10 min. PCR products were subcloned and sequenced according to the methods described above.

Phylogenetic analysis. The deduced amino acid sequences of Japanese flounder Foxl2, FSHR, and LHR were aligned with other vertebrate proteins using CLUSTAL W, version 1.83 [15]. Phylogenetic trees were constructed using the aligned sequences by the neighbor-joining method [16], using the Phylogeny Inference Package (PHYLIP) version 3.573c. See the Supplementary methods online for DDBJ accession numbers of the sequences used for the analysis.

RT-PCR. One microgram total RNA extracted from gonads or pituitaries of the juveniles at several stages using ISOGEN (Nippongene) was reverse-transcribed using RNA PCR Kit (Applied Biosystems) at 42 °C for 30 min. PCR was performed with the gene-specific primer pair using the gonad or pituitary cDNA as a template in the PCR mixture. See the Supplementary methods online for the primer sequences and PCR conditions.

In situ hybridization. Japanese flounder juveniles at 100 dah were fixed in Bouin's solution at 4 °C overnight, dehydrated in graded ethanol, embedded in paraffin, and sectioned serially at 5-μm thickness. *In situ* hybridization was performed with a DIG-labeled *foxl2* or *cyp19a1* RNA probe using the sections as described previously [17].

Plasmids. The Japanese flounder *cyp19a1*-luciferase reporter and the Foxl2 expression plasmids were constructed by ligating the *cyp19a1* promoter into PicaGene Basic Vector 2 (Nippongene) and the *foxl2* cDNA containing the ORF into pcDNA3.1 (Invitrogen), respectively.

See the Supplementary methods online for methods of transient transfection assay and electrophoretic mobility shift assay (EMSA).

Results and discussion

Isolation and characterization of 5′-flanking region of Japanese flounder cyp19a1 gene

The 5′-flanking region of the *cyp19a1* gene isolated from the Japanese flounder was 767-bp in length. Comparing the 5′-flanking region of the flounder *cyp19a1* gene with the 5′-end of the flounder *cyp19a1* cDNA isolated by RACE [4], the *cyp19a1* promoter region was 734-bp in length from the transcription initiation site (DDBJ Accession No. AB303853), and contained a putative TATA box, two Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF1) binding sites (5′-TCAAGGGCG-3′ and 5′-TCAAGGTT A-3′), an estrogen-responsive element half site (ERE-half: 5′-TGACC-3′), a forkhead-responsive consensus site (5′-GCAAACA-3′) and a cAMP-responsive element (CRE)-like sequence (5′-AAACGTCA-3′).

Molecular cloning and gonadal expression analysis of foxl2 in Japanese flounder

The isolated cDNA was 1593-bp long, which contained an ORF encoding a potential protein of 306 amino acid residues. The protein contained a forkhead DNA-binding domain of 110 amino acid residues, which are well conserved among mammalian, chicken, and other fish Foxl2s. Phylogenetic analysis showed that the protein was evolutionarily close to mammalian, chicken and other teleost fish proteins, while it was distant from mouse Foxl1, a member of the forkhead family (Fig. 1A). Therefore, these results suggest that the isolated cDNA is a homolog of *foxl2* in Japanese flounder (*foxl2*: DDBJ Accession No. AB303854).

In the present study, phenotypic females and males were produced by rearing the genetically female flounder at 18 °C and 27 °C, respectively. Expression pattern of *foxl2* mRNA in gonads of both sexes during the sex differentiation period was analyzed by RT-PCR. On 50 (before morphological sex differentiation) and 60 dah (initiation period of sex differentiation) [4], *foxl2* mRNA was expressed weakly in the gonads of the genetically female juveniles reared at 18 °C, whereas it was hardly detected in those reared at 27 °C (Fig. 1B). On 80 and 100 dah, the mRNA