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High temperature induces *cyp26b1* mRNA expression and delays meiotic initiation of germ cells by increasing cortisol levels during gonadal sex differentiation in Japanese flounder

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ABSTRACT

The Japanese flounder (*Paralichthys olivaceus*) is a teleost fish with an XX/XY sex determination system. XX flounder can be induced to develop into phenotypic females or males, by rearing them at 18 °C or 27 °C, respectively, during the sex differentiation period. Therefore, the flounder provides an excellent model to study the molecular mechanisms underlying temperature-dependent sex determination. We previously showed that cortisol, the major glucocorticoid produced by the interrenal cells in teleosts, causes female-to-male sex reversal by directly suppressing mRNA expression of ovary-type aromatase (*cyp19a1*), a steroidogenic enzyme responsible for the conversion of androgens to estrogens in the gonads. Furthermore, an inhibitor of cortisol synthesis prevented masculinization of XX flounder at 27 °C, suggesting that masculinization by high temperature is due to the suppression of *cyp19a1* mRNA expression by elevated cortisol levels during gonadal sex differentiation in the flounder. In the present study, we found that exposure to high temperature during gonadal sex differentiation upregulates the mRNA expression of retinoid-degrading enzyme (*cyp26b1*) concomitantly with masculinization of XX gonads and delays meiotic initiation of germ cells. We also found that cortisol induces *cyp26b1* mRNA expression and suppresses specific meiotic marker *synaptonemal complex protein 3 (sycp3)* mRNA expression in gonads during the sexual differentiation. In conclusion, these results suggest that exposure to high temperature induces *cyp26b1* mRNA expression and delays meiotic initiation of germ cells by elevating cortisol levels during gonadal sex differentiation in Japanese flounder.

1. Introduction

The Japanese flounder (*Paralichthys olivaceus*) is a teleost fish with an XX/XY sex determination system. XX flounders can be reversed to a male phenotype by rearing the larvae at high water temperatures [1–3]. Thus, the sex of the flounder is determined by genotype and temperature effects [4]. Previously, we induced XX fish to predominantly develop into phenotypic females or males by rearing them at 18 °C or 27 °C, respectively, during the sex differentiation period [5]. Therefore, the flounder provides an excellent model to study the molecular mechanisms underlying temperature-dependent sex determination (TSD). We have also demonstrated that fadrozole (an aromatase inhibitor), tamoxifen (an anti-estrogen), and 27 °C treatments induce masculinization of XX flounder by suppressing the mRNA expression of ovary-type aromatase (*cyp19a1*), a steroidogenic enzyme responsible for the conversion of androgens to estrogens in the gonads. Furthermore, masculinization induced by these treatments was inhibited by 17β-estradiol (E2), which suggests that the suppression of *cyp19a1* mRNA expression and the resultant inhibition of estrogen biosynthesis may trigger masculinization of the XX flounder induced by high water temperature [5–7]. More recently, we showed that cortisol, the major glucocorticoid produced by the interrenal cells in teleosts, caused female-to-male sex reversal by directly suppressing *cyp19a1* mRNA expression while metyrapone (an inhibitor of cortisol synthesis) inhibited 27 °C-induced masculinization of XX flounder [8]. These results indicate that masculinization by high temperature is due to the suppression of *cyp19a1* mRNA expression following elevations in cortisol levels during gonadal sex differentiation in the flounder.

Cytochrome P450 aromatase, the product of the *cyp19* gene, is expressed in many tissues and plays an important physiological role in the regulation of estrogen biosynthesis. In non-mammalian vertebrates, the expression of *cyp19* mRNA was reported to be much higher in the ovary than in the testis during gonadal sex differentiation [5,9–11]. Moreover, aromatase inhibitors were shown to induce masculinization in chickens [12], reptiles

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[13–15], amphibians [16], and teleost fishes [6,17,18]. These findings suggest that estrogen plays a pivotal role in ovarian differentiation in non-mammalian vertebrates containing species that undergo TSD. However, it is still unclear what factors are regulated by estrogen during gonadal sex differentiation. Moreover, the mRNA expression of *cyp26b1*, a cytochrome P450 enzyme that metabolizes retinoic acid (RA), was repressed in female gonads during the meiotic stage and in male gonads treated with E2, but not in standard male gonads, in a urodele amphibian with a ZZ/ZW genetic mode of sex determination, suggesting that estrogen may be involved in *cyp26b1* expression in that species [19].

It was reported that CYP26B1 regulates RA signaling during meiotic initiation of germ cells in mice [20,21]. They showed that RA is released from the mesonephros in both sexes from as early as 10.5 days post-conception (dpc), while *Cyp26b1* is initially expressed in gonads of both sexes at 11.5 dpc but becomes highly male-specific by 12.5 dpc. Although mouse germ cells remain very similar in male and female gonads until about 13.5 dpc, at this age, female germ cells enter the prophase of meiotic division in RA-abundant gonads without *cyp26b1* expression, whereas male germ cells arrest in G0 or G1 of the mitotic cycle in RA-poor gonads expressing *cyp26b1*, resuming mitosis after birth [20]. Moreover, inhibition of *Cyp26b1* in the testis induces the expression of specific meiotic markers, whereas blockade of RA signaling pathway in the ovary delays meiosis entry [20]. Hence, CYP26B1 plays a key role in determining whether or not germ cells enter meiosis by controlling local distribution of RA. Recently, it was reported the conserved role of CYP26B1 on meiotic initiation of germ cells in chicks and amphibians, respectively [19,22]. However, it remains unclear whether *cyp26b1* expression is involved in RA-dependent meiosis entry in teleosts.

To elucidate the relationship between *cyp26b1* expression and meiosis onset in teleosts, particularly in species with TSD, we investigated the mRNA expression profiles of *cyp26b1* and a meiosis-specific marker, *synaptonemal complex protein 3 (sycp3)*, in Japanese flounder. In this study, we found that high water temperature induced *cyp26b1* mRNA expression concomitantly with masculinization of XX gonads and delayed meiotic initiation of germ cells. Furthermore, cortisol induced *cyp26b1* mRNA expression and suppressed *sycp3* mRNA expression in gonads during sexual differentiation.

2. Materials and methods

2.1. Animals

All genetically female (XX) broods of Japanese flounder (*P. olivaceus*) were produced artificially by mating the flounder females with sex-reversed males as previously described [5]. Larvae were kept at 18–20 °C for 29 days after hatching (dah). Phenotypic females and males were produced by rearing the XX broods at 18 °C and 27 °C, respectively, from 30 to 100 dah, the critical sex differentiation period in the flounder [5].

2.2. Isolation and sequence analysis of Japanese flounder cyp26b1 and sycp3 cDNAs

One microgram of total RNA was extracted from the adult testes using ISOGEN (Nippongene, Tokyo, Japan) and reverse-transcribed using a RNA PCR kit (Applied Biosystems, Foster City, CA) at 42 °C for 30 min. To isolate Japanese flounder *cyp26b1* and *sycp3* cDNAs, PCR was performed with the specific primers (*cyp26b1*: 5′-GTGTCCCAGCAGCTGTGGCAGCT-3′ and 5′-ACCAAAGGGCAGGTAGTGAAAGC-3′; *sycp3*: 5′-AGAAACTGGAGCAGCTGTGG-3′ and 5′-ACTTGCGGACAGTGGCCATCTC-3′), which were designed based on the conserved sequences from other animal CYP26B1 or SYCP3 proteins, using the testicular 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), and 1 unit Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems)]. The 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 and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The 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) using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems).

2.3. Phylogenetic analysis

The deduced amino acid sequences of Japanese flounder CYP26B1 and SYCP3 were aligned with other vertebrate proteins using CLUSTAL W software, version 1.83 [23]. Phylogenetic trees were constructed using the aligned sequences by the neighbor-joining method [24], using the Phylogeny Inference Package (PHYLIP) version 3.573c. See the Supplementary methods online for the DDBJ accession numbers of the sequences used for the analysis.

2.4. Gonadal organ culture technique

Gonadal organ culture was performed using XX gonads at 55 dah as previously described [8]. The gonads were replaced on nucleopore filters (Whatman, Tokyo, Japan) in 35 mm plastic dishes (Falcon, Los Angeles, CA), and then cultured in 2 ml of the basal culture medium with or without cortisol (10^{-6} M) at 18 °C for 2 weeks. The basal culture medium consisted of Leibovit's L-15 medium (Gibco BRL) supplemented with 0.5% bovine serum albumin and 10 mM Hepes (pH 7.4).

2.5. RT-PCR

RT-PCR was performed as previously described [25] with the following primers (forward and reverse): *cyp26b1* (DDBJ accession No. AB693197), 5′-TGCCCATGCCGAAAGGATCC-3′ and 5′-TTGGGAATCTGGACTCCGTC-3′; *sycp3* (DDBJ accession No. AB693198), 5′-TGGAGCAGCTGTGGAGCAAC-3′ and 5′-ATCTCCTGCTGTTGTGTGTC-3′; *cyp19a1* (DDBJ accession No. AB017182), 5′-ATCGGATCCCTGCCTGTGAC-3′ and 5′-TGGCTGATGCTCTGCTGAGG-3′; *Müllerian inhibiting substance* (*mis*; DDBJ accession No. AB166791), 5′-TGACCCGTACCTACGAGCTG-3′ and 5′-TCGTCCACGTTCTCGCTCTC-3′; *elongation factor 1α* (*ef-1α*; DDBJ accession No. AB017183), 5′-AGTTCGAGAAAGAAGCTGCC-3′ and ATCCAGAGCATCCAGCAGTG-3′. The PCR conditions were as follows: preheating at 95 °C for 10 min, 25 (*ef-1α* and *mis*) or 30 cycles (*cyp26b1*, *sycp3* and *cyp19a1*) of PCR at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min.

2.6. Real-time PCR

The *sycp3* mRNA levels were determined by quantitative real-time PCR using SYBR Green I Master (Roche Diagnostics) on a LightCycler 480 (Roche) with the primers described above. The PCR conditions were as follows: preheating at 95 °C for 5 min, and 45 cycles of PCR at 95 °C for 10 s, 55 °C for 10 s and 72 °C for 10 s.

2.7. Histology

Japanese flounder juveniles at 100 dah were fixed in Bouin's solution at 4 °C overnight, dehydrated in graded ethanol, embed-