wnt4 Is Associated with the Development of Ovarian Tissue in the Protandrous Black Porgy, Acanthopagrus schlegeli

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ABSTRACT

The protandrous black porgy, Acanthopagrus schlegeli, has a striking life cycle with sex differentiation at the juvenile stage, mono-male development, and male-to-female sex change (with vitellogenic oocytes) at age 3 yr. In the present study, we investigated the possible roles of wnt4 in gonadal development in a nonmammalian model organism (protandrous black porgy), especially in relation to sex differentiation, ovarian growth, and sex change. Fish of various ages were treated with estradiol (E2) or aromatase inhibitor (AI) to determine whether manipulation of the hormonal environment had an effect on these processes. Furthermore, a natural sex change (≥2-yr-old fish) and a nonchemical method to induce an early sex change (≥1-yr-old fish) via the removal of testicular tissue were examined in this study. We present herein an integrative immunohistochemical, cellular, and molecular data set describing these phenomena. During gonadal sex differentiation, no increase in wnt4 expression was detected. A profile of increased wnt4 expression and decreased cyp19a1a expression was associated with ovarian growth (proliferation of oogonia and development of ovarian lamellae) in ≥1-yr-old fish. Both E2 and AI induced an increase in wnt4 transcripts and resulted in ovarian development in 0-yr-old and ≥1-yr-old fish. Increased wnt4 transcripts were found in ovarian tissue undergoing development from primary oocytes to vitellogenic oocytes during the natural sex change in ≥2-yr-old fish. Removal of testicular tissue in ≥1-yr-old fish resulted in successful early sex change (with vitellogenic oocytes) 6 mo after the excision. During the process of the early sex change (3 mo after testis excision), the fish ovary became active and had increased diameter of the primary oocytes; this was in accord with increased ovarian wnt4 expression but not sf1, foxl2, and genes in the steroidogenic pathway, including cyp19a1a. Wnt4 staining further confirmed the profile of wnt4 expression associated with ovarian development. The results of the present study suggest that wnt4 has important roles in late ovarian growth (e.g., oogonia proliferation and structure of ovarian lamellae) and the natural sex change (vitellogenic oocytes) in the protandrous black porgy.

INTRODUCTION

Black porgy, Acanthopagrus schlegeli Bleeker, are marine protandrous hermaphroditic fish that have a striking life cycle. The fish are functional males for the first 2 yr of life, but they transform into females during the third year [1, 2]. A differentiated bisexual gonad divided by connective tissue is observed in 4- to 5-mo-old fish [3]. The testicular tissue further develops to become a functional testis for the first and second reproductive seasons. The ovarian compartment contains few oogonia and primary oocytes in the germinal epithelium around the ovarian cavity between sex differentiation (4-mo-old fish) and the first reproductive season [3]. During the postspawning season (March to May) and nonspawning (June to August) periods after the first reproductive season, the testicular tissue regresses, and the ovarian tissue expands to become the dominant tissue (with ovarian lamellae) in the bisexual gonad, although the germ cells remain at the primary oocyte stage [3]. The ovarian tissue continues to be the predominant tissue in the bisexual gonad until the prespawning season (September to December) in ≥1-yr-old fish, at which point it regresses concomitantly with the growth of testicular tissue. The testicular tissue resumes dominance and activity for the second reproductive season. A similar pattern of ovarian development is observed during the postspawning and nonspawning seasons in ≥2-yr-old fish, but the ovarian tissue does not regress in about 40%–50% of the population. The fish will subsequently change sex by developing vitellogenic oocytes in the approaching third reproductive season [3, 4]. Plasma estradiol (E2) levels during the late prespawning and spawning periods are correlated with the natural sex change in ≥2- to 3-yr-old fish [1, 2], and the natural sex change is blocked by long-term aromatase inhibitor (AI) administration [5]. Plasma E2 is maintained at low levels, however, when ovarian tissue develops to become the dominant tissue (with ovarian lamellae) during the postspawning and nonspawning seasons in ≥1-yr-old and ≥2-yr-old fish [2, 4, 6]. Furthermore, ovarian tissue is arrested at the primary oocyte stage until the sex change [2, 5, 7]. In our recent investigations, cyp19a1a transcript levels were high before male sex differentiation and dramatically decreased after the testis underwent early spermatogenesis [8, 9]. Testicular tissue development was blocked by long-term AI administration, and ovarian tissue developed after testis regression in juvenile fish [9]. As such, it would be relevant to understand the mechanisms behind ovarian development (with ovarian lamellae) in a low E2 environment.

Few species have been studied in relation to Wnt4 (wingless-type MMTV integration site family, member 4) in gonad development. Wnt4 has been reported to be related to ovarian development in mice [10]. It induces the expression of downstream genes such as Fst and has been observed to exhibit sexually dimorphic expression and is essential for the development of mouse ovaries [11]. Wnt4-mutant females
show a lack of Mullerian ducts, an increase in ectopically steroidogenic cell migration, and the activation of testosterone biosynthesis [10, 12]. Wnt4 is predominantly expressed in the ovary in mammals [10] but has no sexually dimorphic expression during female differentiation of nonmammalian vertebrates, including chickens [13], turtles [14], and frogs [15]. The profile and possible roles of wnt4 expression in relation to gonad development are not yet clear in teleost fish.

We propose that Wnt4/wnt4 has an important role in ovarian development, especially in relation to early formation of ovarian lamellae and the late sex change in the protandrous black porgy. In the present study, protandrous black porgy exhibiting unique development of a bisexual gonad and a natural sex change were chosen to study wnt4 profiles in relation to the following: 1) gonadal sex differentiation (i.e., from undifferentiated to differentiated juveniles \([\geq 0\text{-yr-old fish}]\) given the functional mono-male sex pattern observed in this species during the first and second reproductive seasons, 2) E2 activity during sex differentiation and gonad development using exogenous E2 and AI treatments in \(>0\text{-yr-old fish and AI treatment in} >1\text{-yr-old fish}, 3)\) the natural sex change from male to female in \(\geq 2\) to 3-yr-old fish, and 4) the testicular effects on the development of ovarian tissue in the bisexual gonad and the expression of wnt4 by in vivo surgical manipulation of testicular tissue and ovarian tissue.

**MATERIALS AND METHODS**

**Experimental Fish**

Three batches of black porgy, *A. schlegeli*, were obtained for the experiments. Two-month-old fingerlings \((n = 270, >0\text{-yr-old fish}[\text{mean } \pm \text{SEM}, 1.53 \pm 0.20 \text{ g body weight} 4.62 \pm 0.17 \text{ cm body length}]) were obtained from a pond culture in April for examination of sexual differentiation and the experiment involving E2 and AI administration. Six-month-old differentiated black porgy \((n = 60, [\text{mean } \pm \text{SEM}, 75.9 \pm 4.3 \text{ g body weight}])\) were obtained in September to study the effects of various doses of E2 on gonadal development. The \(>1\text{-yr-old black porgy} (n = 100, [\text{mean } \pm \text{SEM}, 23.86 \pm 1.65 \text{ g body weight} 11.67 \pm 0.26 \text{ cm body length}])\) were obtained in March for study of ovarian development and AI administration, while another batch \((n = 40, [\text{mean } \pm \text{SEM}, 82.00 \pm 2.69 \text{ g body weight} 16.60 \pm 0.21 \text{ cm body length}])\) was used for testis excision. Two-year-old black porgy \((n = 70, [\text{mean } \pm \text{SEM}, 363.93 \pm 18.19 \text{ g body weight} 26.6 \pm 0.32 \text{ cm body length}])\) were obtained in February to study E2 injection and the natural sex change and gene profiles during the annual reproductive cycle. Total body weight and gonadal weight were measured for calculation of the gonadosomatic index \((\text{gonadal weight} / \text{body weight}) \times 100\%\).

The experimental fish were acclimated to the pond environment at the university culture station in seawater and a natural lighting system. Water temperatures ranged from 19 to 26°C. The fish were fed commercial feed (Fwa Sou Feed Co., Taichung, Taiwan) ad libum. All procedures and investigations were approved by the National Taiwan Ocean University institutional animal care and use committee and were performed in accord with standard guiding principles.

**Experimental Design**

**Experiment 1: gene profile during gonadal differentiation in >0-yr-old fish.** Sexually undifferentiated juveniles were obtained to examine the expression profile and morphological changes in gonads during differentiation. Fish \((>0\text{-yr-old})\) were collected regularly \((n = 8-20 \text{ fish})\) in June \((3\text{-mo-old undifferentiated gonad}), July (4\text{-mo-old differentiating gonad}), and August (5\text{-mo-old differentiated gonad}). Gonad samples were collected to examine the status of gonads and for genetic analyses.

**Experiment 2: gene profiles during E2- and AI-induced female sex differentiation in >0-yr-old undifferentiated fish.** To further clarify the importance of E2 and cyp19a1a/cyp19a1a for testicular differentiation, we conducted E2 and AI treatments in undifferentiated fish. Undifferentiated fish \((3\text{-mo-old})\) were divided equally into the following three groups in June 2005: control, E2 treated, and AI treated. Estradiol (Sigma, St. Louis, MO) and AI \((1,4,6\text{-androstatrien}-3, 17\text{-dione}; \text{Steroids Inc., Newport, RI})\) were dissolved in ethanol and mixed with feed and water to obtain a final concentration of 6 mg/kg of feed for E2 and 20 mg/kg of feed for AI. The treatments were administered from June to January of the following year \((7 \text{ mo})\). The control group was fed with feed devoid of E2 or AI. Fish were collected in September \((6\text{-mo-old differentiated gonad})\) for expression analyses \((n = 8 \text{ fish per group})\), histology \((n = 6 \text{ fish per group})\), and blood analysis \((n = 8 \text{ fish per group})\). Estradiol measurements were performed only in 6-0-yr-old fish.

**Experiment 3: the effects of E2 on the morphological changes and gene profile in the gonads of >0-yr-old differentiated fish.** To further clarify the effects of E2 on gonadal development, gonadal differentiated fish \((5\text{-mo-old})\) were equally divided into the following two groups in July: control and E2 treated \((6 \text{ mg/kg of feed})\). Estradiol-treated fish were fed an E2 diet from July to October, and E2 administration was withdrawn and replaced by the control diet \((\text{devoid of E2})\) from November to January of the following year. Fish samples \((n = 8 \text{ fish per group})\) were collected monthly for genetic analysis and histology.

**Experiment 4: the effects of endogenous E2 on the morphological changes and gene profile in the gonads of >1-yr-old differentiated fish.** To examine the effects of E2 on testicular and ovarian development in sexually differentiated fish, >1-yr-old differentiated black porgy were divided into the following two groups \((n = 50 \text{ fish per group})\) in March 2008: control and AI treated \((20 \text{ mg/kg of feed})\). Fish gonads were collected in April and June \((1 \text{ mo})\) and 3 mo after treatment, respectively for expression analyses \((n = 8 \text{ fish per group})\), histology, and immunohistochemical staining with proliferating cell nuclear antigen (PCNA) antiserum \((n = 6 \text{ fish per group})\). Gonadal E2 concentrations in June were measured using an E2 enzyme immunoassay.

**Experiment 5: gene profiles during the natural sex change in >2- to 3-yr-old fish.** To examine the expression profile in gonads during the natural sex change, >1-yr-old black porgy were collected regularly \((n = 8 \text{ fish})\) at 1 mo or 2 mo from March 2007 \((\text{first spawning})\) to January 2008 \((\text{second spawning season})\). Two-year-old black porgy \((n = 8 \text{ fish})\) were collected in January 2008 \((2 \text{ mo after the second spawning})\) to January 2003 \((\text{after the sex change})\). Ovarian samples were collected for examination of sex status \((\text{male vs. female})\) and for genetic analyses.

To localize wnt4/Wnt4 expression, >1-yr-old fish \((\text{March to May after spawning season})\) and >2-yr-old fish \((\text{November before spawning season})\) were collected for in situ hybridization and immunohistochemical staining.

**Experiment 6: testis excision from the bisexual gonad in >1-yr-old fish.** To investigate the interaction between testicular and ovarian tissue in the bisexual gonad, >1-yr-old fish were divided into testis-excised and sham groups in June. Using in vivo surgical methods, testicular tissue was carefully and completely removed from the bisexual tissue \((\text{keeping the ovarian tissue intact})\). The fish were deeply anesthetized in 2-phenoxethanol solution \((0.5 \text{ ml/L})\) (Chowa Chemical, Tokyo, Japan). After surgery, the ligature with interrupted skin sutures was applied. The sham group was subjected to surgery but did not undergo removal of the testis \((\text{intact bisexual gonad})\). Gonads were collected in September \((n = 8 \text{ fish per group})\) and January \((n = 8 \text{ fish per group})\) of the next year to examine gene expression and gonad histology.

**Experiment 7: short-term effects of E2 on the genetic profile in ovarian tissue from >2-yr-old fish.** To examine the short-term effects of E2 on the ovarian genetic profile, >2-yr-old black porgy were equally divided into the following three groups \((n = 10 \text{ fish per group})\) in October 2001: control, low-dose E2 \((50 \text{ ng/g of body weight})\), and high-dose E2 \((1.5 \text{ mg/g of body weight})\). Estradiol was injected \((i.p.)\) on Day 0 and Day 5, and fish gonads were collected on Day 6 for genetic and histological analyses. No histological changes in the gonads were observed as a result of this short-term E2 treatment.

**Genes Studied Based on Known Roles in Gonad Development**

It has been reported that fost2 is related to cyp19a1 and ovarian development [16, 17]. Steroidogenesis-related factors such as mres1a (g1 [an essential factor 1] or adrenal 4 binding protein], dax-1 (a sex-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X-chromosome, gene 1]), or star (steroid acute regulatory protein) and enzymes such as cyp11a1 (cytochrome P450 side chain cleavage enzyme), cyp17a1 (17a-hydroxylase/C17-20 lyase), cyp19a1a (aromatase), and cyp11b2 (11b-hydroxylase) were measured to study changes in expression with respect to gonadal activity and wnt4 expression. The germ-cell-specific gene vasx [18] and oocyte-specific gene figla [19] were used to identify germ cell numbers and oocyte-specific, respectively, and were monitored in relation to gonadal differentiation.

**Quantification of Gene Transcripts by Quantitative Real-Time PCR Analysis**

One microgram of total RNA extracted from gonad of the representative fish was reverse transcribed to the first-strand cDNA using Superscript II (Invitrogen, Carlsbad, CA) with the oligo (dt)20-28 primers. The first-strand
cDNA was used for the quantitative real-time PCR analysis. Quantitative real-time PCR analysis of gene transcripts of wnt4 (DQ266468), fox2a (ACAA79579), cyp19a1a (P450arom, AY870246), cyp11b2 (P45011b1, EF 423618), nr5a1 (AY491379), nr0b1 (EF423617), star (AY870248), cyp11a1 (AY870246), cyp17a1 (AY870249), and glyceraldehyde-3-phosphate dehydrogenase (gapdh, DQ99798) was conducted according to the methods described in previous studies [8, 9, 20]. Specific primers for wnt4 (sense 5′-GGAGCAATGCGAGGTATGC-3′ and antisense 5′-TCACCTCACACTGCGCTTA-3′) and other genes [8, 9] were designed for the quantitative real-time PCR. Quantification of standards, samples, and controls were conducted simultaneously by real-time PCR (GeneAmp 5700 Sequence Detection System; Applied Biosystems, Foster City, CA) with SYBR green I as a double-stranded DNA minor groove binding dye. The standard curves of log (transcript concentrations) vs. C_{t} (the calculated fractional cycle number at which the PCR-fluorescence product is detectable above a threshold) were obtained. The correlation of the standard curve was >0.999. The values detected from different amounts of RNA (10-fold dilution series) of the representative samples aligned in parallel with the respective standard curve. The transcript values of each gene were calibrated with internal control gapdh and then normalized (× 100%).

Gonadal Histology, wnt4 In Situ Hybridization and Immunohistochemical Staining with WNT4 and PCNA Antisera

A piece of the gonad tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 or 5 μm. Transverse sections were subjected to hematoxylin-eosin, in situ hybridization, or immunohistochemical staining. Gonadal developmental stages were determined.

For wnt4 in situ hybridization, the digoxigenin-labeled RNA probes, both sense and antisense, were produced from plasmids containing the cDNA of the wnt4 gene using T7 or SP6 transcriptase (Promega, Madison, WI). The sections were rehydrated in a sodium phosphate buffer with saline (PBS) and then incubated in sense or antisense digoxigenin-labeled RNA probes with hybridization buffer (50% formamide, 5× saline-sodium citrate, 500 μg/ml of tRNA, 50 μg/ml of heparin, and 0.1% Tween-20 [pH 6.0]) at 60°C overnight. Alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche, Penzberg, Germany) was absorbed in acetone powder from gonad at room temperature for 1 h. Immunostaining was performed with preabsorbed antibodies at room temperature for 1 h, with the blocking buffer containing 2% blocking reagent (Roche, Mannheim, Germany) and 20% sheep serum. After washing, NTMT (100 mM NaCl, 100 mM Tris-HCl, 50 mM MgCl_{2}, and 0.1% Tween-20 [pH 9.5]) was used to ensure the pH is high. Finally, the NBT/BCIP Detection System (Sigma) was used to detect wnt4 expression.

For immunohistochemical staining, the section was rehydrated in a PBS buffer (pH 7.6) and incubated with 3% H_{2}O, in PBS. The section was then incubated with 1.5% normal goat serum for 30 min and with antiserum against human PCNA (sc-7907; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or antisera against human WNT4 (sc-13962; Santa Cruz Biotechnology, Inc.) overnight at 4°C. This was followed by incubation with biotinylated anti-rabbit IgG (H + L; Vector Laboratories Inc., Burlingame, CA). Color formation was amplified with an ABC kit (avidin-biotin; Vector Laboratories Inc.) and DAB (3,3′-diaminobenzidine; Sigma). PCNA antiserum was induced in the rabbit against human PCNA, which has 92% amino acid identity with the black porgy. WNT4 antisera was induced in the rabbit against human WNT4, which has 82% amino acid identity with the black porgy. The specificity of WNT4 and PCNA antiserum was evaluated by Western blotting. Immunoblotting was performed with preabsorbed antibodies at 4°C overnight, with the blocking buffer containing 0.5% nonfat dry milk. After washing, nitrocellulose membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham, Freiburg, Germany) at 4°C overnight. Finally, the ECL Plus Western Blotting Detection System (Amersham) was used to detect protein staining. A specific and single protein band corresponding to the black porgy PcnA and wnt4 (according to the molecular weight) was detected.

Measurement of E2 by Enzyme Immunoassay

Gonad tissue was homogenized with a potassium phosphate buffer and centrifuged at 10000 g for 10 min at 4°C. Plasma and tissue homogenates were extracted with ethyl ether and then measured with an enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI).

Statistical Analysis

All data are expressed as the mean ± SEM. The values were subjected to analysis by one-way ANOVA, followed by Student-Newman-Keuls multiple test, with P < 0.05 indicating significant difference. Student t-test was also performed to determine significant differences (P < 0.05) between treatments.

RESULTS

Gene Profiles During Early Gonadal Development in ≥0-Year-Old Fish (Experiment 1)

To determine which genes were expressed during gonadal sex differentiation, a real-time PCR assay was performed in the gonad during early gonadal development at age 3 mo (undifferentiated) to age 5 mo (differentiated). A germ cell-specific gene, vasa, was unchanged in the gonad at age 3 mo and 4 mo and then significantly increased in 5-mo-old fish (Fig. 1). An oocyte specific gene, figla, was dramatically increased at age 4 mo after ovarian cavity formation (Fig. 1). Also, wnt4 and fox2 were unchanged in the gonad at age 3–5 mo during early gonadal development (Fig. 1). Moreover, cyp19a1a was maintained at high levels at age 3 mo and 4 mo but decreased significantly at age 5 mo (Fig. 1). A male-specific gene, dmrt1, was dramatically increased at age 5 mo during testicular development (Fig. 1).

Ovarian Development Is Not Only Stimulated by E2 Administration but Also Induced by AI Administration in Undifferentiated ≥0-Year-Old Fish (Experiment 2)

Estradiol and AI were given to undifferentiated fish. After 3 mo of E2 administration and 4 mo of AI administration, the ovarian lamellae became branched, hyperplastic, and protruded into the ovarian cavity. Ultimately, the fish had well-developed ovarian tissue and regressed testicular tissue. After 3 mo of treatment, plasma E2 concentrations in 6-mo-old fish were 58.8 ± 3.5, 154.0 ± 12.8, and 40.0 ± 4.0 pg/ml in the control, E2 group, and AI group, respectively (P < 0.05).

Based on immunohistochemical staining, there was no Wnt4 protein expression in the control (gonad mainly with testicular tissue) (Fig. 2a). In the ovarian tissue of E2- and AI-induced females, Wnt4 protein was expressed in the early...
follicle cells around the oogonia and ovarian cortex but dramatically decreased in the follicle cells at the primary oocyte stage in September (Fig. 2, b and c). More intense Wnt4 staining was found in the E2-treated fish in September (Fig. 2b) compared with the AI-treated fish (Fig. 2c).

Increased levels of \(wnt4\) transcripts were found in the E2- and AI-induced ovarian tissue at ages 5 mo and 6 mo (August and September, respectively [2 and 3 mo, respectively, after treatment]) compared with the gonad in the control group (Fig. 2d). Estradiol caused higher \(wnt4\) transcript expression than AI in September (Fig. 2d).

**Wnt4 Expression Was Decreased after Long-Term E2 Administration in E2-Stimulated Ovarian Tissue in Differentiated \(\geq 0\)-Year-Old Fish (Experiment 3)**

After 1 mo of E2 administration, the development of ovarian tissue was detected (in August) in fish at age 6 mo (Fig. 3a). Many primary oocytes were found in the ovarian tissue at age 7 mo (Fig. 3b). After 3 mo of E2 administration, the fish exhibited completely regressed testicular tissue and well-developed ovarian tissue with ovarian lamellae, and ovarian lamellae became branched and hyperplastic and protruded into the ovarian cavity (Fig. 3c). Estradiol administration was withdrawn (E2 termination) after October, and fish were fed a control diet from November to January. These E2-stimulated early sex change fish had a reversible sex change after E2 termination. After E2 termination, testicular tissue regenerated in concert with the regression of ovarian tissue, and the gonad became bisexual in January (Fig. 3d). Testicular tissue was the primary tissue in the gonad in the \(\geq 0\)- to 1-yr-old control fish (data not shown).

Quantitative real-time PCR data showed that \(wnt4\) transcripts remained low in the testicular tissue in the control group compared with the ovarian tissue in the E2-treated and E2-terminated groups (Fig. 3e). After 1 mo of E2 administration (August), \(wnt4\) was expressed in E2-induced ovarian tissue and significantly increased after 2 mo of E2 administration...
(September) but dramatically decreased after 3 mo of E2 administration (October) (Fig. 3e). The expression levels of wnt4 did not change significantly after E2 termination during the period from November to December when ovarian tissue was less active compared with the fully developed ovary after 3 mo of E2 administration (October) (Fig. 3e). The wnt4 transcripts remained low in the bisexual gonad of the E2-terminated group in January (Fig. 3e).

Treatment with AI Resulted in Increased Ovarian Development and wnt4 Expression in ≥1-Year-Old Fish (Experiment 4)

Immunohistochemical staining for PCNA revealed that highly proliferating ovarian tissue was observed in both control and AI-treated ovarian tissue (Fig. 4). However, stronger PCNA staining in many cells was found in the testicular tissue of control fish vs. few PCNA-positive cells in the AI-treated group (Fig. 4). Ovarian tissue was better developed in the AI-treated fish compared with the control (histological data not shown).

After 4 mo of AI administration in ≥1-year-old fish, the gonadosomatic indexes in the control and AI groups in July were 0.070 ± 0.013 and 0.040 ± 0.009, respectively (P > 0.05). Gonadal E2 concentrations were 18.26 ± 1.51 pg/g of gonad and 1.38 ± 0.26 pg/g of gonad in the control and AI groups, respectively (P < 0.05).

Quantitative real-time PCR analyses showed that higher levels of wnt4 were found in the AI-treated gonads after 1 mo and 3 mo of AI administration compared with the expression level in controls. These results are shown in Figure 4.

Testicular cyp11b2 and Ovarian wnt4 Expression Profiles in ≥1- to 3-Year-Old Fish During the Natural Sex Change (Experiment 5)

Quantitative real-time PCR data showed that transcripts of a testicular spermatogenesis-related gene, cyp11b2 [8, 21], were low during the nonspawning season and significantly increased during the nonspawning and prespawning seasons (August to November) in ≥1-year-old fish (Fig. 5). Expression of cyp11b2 was significantly decreased during the second spawning season; low expression levels were maintained in the postspawning and nonspawning seasons and remained low during the prespawning season in ≥2-year-old fish and the natural sex change (Fig. 5). Conversely, ovarian wnt4 expression was high in May during the nonspawning season when ovarian tissue had just begun to develop, and significantly decreased after the nonspawning season (June to January) in ≥1-year-old fish (Fig. 5). Ovarian wnt4 expression was high again in May in ≥2-year-old fish when ovarian tissue started to develop. It was increased in ≥2-year-old fish
(September to January) and reached its highest levels in the third spawning season during the natural sex change (Fig. 5).

**Wnt4 Expression Is Dependent upon the Stage of Oocytes (Experiment 5)**

In situ hybridization demonstrated that *wnt4* transcripts were expressed in the follicle cells of oogonia (Fig. 6a). Immunohistochemical analyses of Wnt4 protein revealed that it was highly expressed in follicle cells surrounding oogonia and early primary oocytes (Fig. 6b, b’ and b’’), but became negative in the follicle cells around the late primary oocytes (Fig. 6b, b and c). Positive Wnt4 staining was also found in the granulosa cells at the early vitellogenic oocyte stage during the natural sex change (Fig. 6c).

**Development of the Sex Change after Removal of Testicular Tissue in the Bisexual Gonad of ≥1-Year-Old Fish (Experiment 6)**

Primary oocytes were observed in September (3 mo after surgery), and the oocyte diameter was significantly different in the control and testis-excised groups (33.20 ± 1.24 and 48.83 ± 2.90 μm, respectively [P < 0.05]). The vitellogenic oocytes and mature oocytes were observed in January in the testis-excised group in 2-yr-old fish (6 mo after testis excision). Few primary oocytes (21.15 ± 1.54 μm) were observed in the control group (as functional males) in January. Based on immunohistochemical staining for PCNA, stronger staining was observed in many oogonia in the testis-excised ovary in September (Fig. 7b) compared with the control (Fig. 7a).

The expression of *foxl2*, *nr5a1a*, *nr0b1*, *star*, and steroidogenic enzymes such as *cyp11a1*, *cyp17a1*, and *cyp19a1a* were all unchanged in the ovarian tissue after excision of testicular tissue in September (3 mo after testis excision) as shown by real-time PCR (Fig. 7c). Only ovarian *wnt4* transcripts were significantly increased (2.4-fold) in the testis-excised group compared with the control group in September (Fig. 7c).

**Increased Expression of wnt4 and foxl2 but Not cyp19a1a with Short-Term E2 Administration (Experiment 7)**

Quantitative real-time PCR data showed that *wnt4* and *foxl2* expression was significantly increased in a dose-dependent

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**FIG. 4.** Relative *wnt4* and *foxl2* expression measured by real-time PCR in ovarian tissue after 1 mo (April) and 3 mo (June) of AI administration. The values were calibrated with the internal control *gapdh*. The control value after 1 mo of AI treatment was defined as 100%. Each value was expressed as the mean ± SEM. *Significant difference (P < 0.05) between the control and AI-treated fish.

**FIG. 5.** Relative expression profiles of *cyp11b2* in testicular tissue and *wnt4* in ovarian tissue during the natural sex change from ≥1- to 3-yr-old fish. The values measured by real-time PCR were calibrated with the internal control *gapdh*, with the value in March of the ≥1-yr-old fish defined as 100%. Each value was expressed as the mean ± SEM. Different letters represent statistical differences (P < 0.05) between seasons (n = 8 for each value).
manner following E2 injection, but *cyp19a1a* expression was not stimulated by E2 treatment. These results are shown in Figure 8.

**DISCUSSION**

Black porgy, with gonadal (testicular and ovarian) sex differentiation in the juvenile stage, mono-male growth and maturation, a bisexual gonad, and a subsequent natural sex change to female, provide a unique model system to study the mechanisms of ovarian development. In the present study, we report (for the first time to our knowledge in teleosts) the expression and important role of *wnt4* in early ovarian development and the later natural sex change in black porgy.

The sex of eutherian mammals is genetically controlled and is completely insensitive to sex steroids such as estrogens and androgens; in marsupials, birds, reptiles, amphibians, and fish, estrogens control gonadal sex, especially the fate of the ovary [17]. Administration of estrogen resulted in the conversion from male to phenotypic female in marsupials [22], birds [23], reptiles [24], and teleosts [25, 26]. Treatment with AI, which blocks aromatase action, also resulted in the production of phenotypic males from females in birds [27, 28], reptiles [29, 30], and teleosts such as tilapia [31], rainbow trout [32],

![Figure 6](image-url)

**FIG. 6.** a) In situ hybridization of *wnt4* in March (postspawning season) in >1-yr-old fish with an antisense probe (a) and with a sense probe (a”). b and c) *Wnt4* immunostaining (brown) in different stages of oocytes. Strong staining is seen in oogonia and early primary oocytes in May (≥1-yr-old fish in postspawning season) (b and c”). No positive staining is seen in the late primary oocytes (b and c). Strong staining is seen in an early vitellogenic oocyte in November (≥2-yr-old fish in prespawning season), counterstained with hematoxylin (purple) in the gonad (c). OG, oogonia; PO, primary oocyte; VO, early vitellogenic oocyte. Positive staining is indicated by arrows. *Oogonia with positive staining.

![Figure 7](image-url)

**FIG. 7.** Cellular proliferation and relative gene profiles of *wnt4*, *foxl2*, *nr5a1a*, *nr0b1*, and genes in the steroidogenic pathway (*star*, *cyp11a1*, *cyp17a1*, and *cyp19a1a*) in the ovarian tissue of >1-yr-old fish in the control and testis-excised groups. Testicular excision was conducted in June, and the fish were sampled in September (3 mo after surgery). a and b) Transverse sections of the gonadal tissue were immunohistochemically stained for Pcna (brown) and counterstained with hematoxylin (purple). c) Relative gene expressions in ovarian tissue measured by real-time PCR (≥3 mo after surgery). The values were calibrated with the internal control *gapdh*, with the control value as 100%. Each value was expressed as the mean ± SEM. *Significant difference (P < 0.05) between the control and AI-treated fish. Pcna is expressed in G1/S phase of the cell cycle, providing evidence for cell cycling activity. OG, oogonia; PO, primary oocyte.
medaka [33], and protandrous black porgy [5]. The importance of E2 in ovarian development was also previously demonstrated in a natural sex change using elevated plasma E2 levels [1]. Thus, endogenous estrogens are traditionally considered important for female sexual differentiation and development [34]. However, ovarian tissue still developed to become a dominant tissue in the bisexual gonad when E2 levels were low in 0-yr-old fish [35]. Furthermore, ovarian tissue developed in the present study when testicular tissue regressed by application of AI. It is also notable that the effects of E2 on gonadal development were dependent upon the E2 dosage: low doses (0.25–1.0 mg/kg of feed) favored testicular growth, while high doses (4–6 mg/kg of feed) favored ovarian growth [35]. It remains unclear how and why ovarian tissue in the bisexual gonad remains inactive, with primary oocytes as the most advanced stage during the first 2½ yr of life just before the natural sex change.

Aromatase inhibitor treatment in undifferentiated ≥0-yr-old fish and differentiated ≥1-yr-old black porgy resulted in low levels of cyp19a1a/Cyp19a1a and E2, in addition to causing testicular regression and further ovarian development. These data indicate the following four findings: 1) ovarian development can occur in an environment with low E2 levels, 2) testicular development is also dependent on E2 support, 3) E2 and AI can similarly result in ovarian growth, and 4) there is an interaction between the development of ovarian and testicular tissues. According to the present data, it is also likely that other factors in addition to E2 are involved in ovarian development in black porgy.

An important characteristic event of the female gonad is the formation of the ovarian cavity, which was observed in 4- to 5-mo-old fish [3]. Based on quantitative real-time PCR data, transcription levels of a germline-specific gene, vasa [18], were no different during ovarian cavity formation. However, an oocyte-specific marker, fig1a [19], was significantly increased after ovarian cavity formation. These data show that ovarian differentiation occurred during ovarian cavity formation in 4- to 5-mo-old fish. Cyp19a1a expression was stronger in the undifferentiated gonad than in the differentiated ovary [9]; however, the ovarian differentiation-related genes wnt4 and foxl2 were unchanged during ovarian differentiation. These data show that wnt4, foxl2, and cyp19a1a did not correlate with ovarian differentiation in black porgy.

Wnt4 has long been considered important for female sexual differentiation in mammals. The expression of Wnt4 is higher in ovarian tissue than in testicular tissue during sex differentiation in mice [10] and marsupials [36], and the loss of Wnt4 leads to partial female-to-male sex reversal in mice [10]. In Wnt4-mutant females, ectopic formation of coelomic vessels in XX gonads occurs through the normal male mechanisms of migration of endothelial cells from the mesonephros into the gonad and overproliferation of steroidogenic cells [12, 37]. These data indicate that normal function of Wnt4 is needed to repress aspects of the male pathway by blocking the migration of endothelial cells into XX gonads and repressing the proliferation of steroidogenic cells [38]. To clarify this issue, wnt4 expression patterns were examined in black porgy. The expression of wnt4 was unchanged during early ovarian development and was unrelated to sex differentiation in juvenile black porgy. Expression of wnt4 was high only during the proliferation of oogonia and at the beginning of the development of ovarian lamellae in ≥0-yr-old (AI-treated and E2-treated fish), ≥1-yr-old (control and AI-treated fish), and ≥2-yr-old (control fish) black porgy. In addition, wnt4 expression in black porgy was higher in females than in males. In other nonmammalian vertebrates (e.g., in chicken [13], turtle [14], and frog [15]), wnt4 transcripts do not demonstrate sex dimorphic expression during gonadal differentiation. High levels of cyp19a1a expression in undifferentiated gonad showed that steroidogenic cells had been migrating into gonad before gonadal differentiation in juvenile black porgy. The finding that steroidogenic enzymes are expressed before gonadal differentiation in black porgy and other nonmammalian vertebrates suggests that wnt4 function is not related to inhibition of the migration and proliferation of steroidogenic cells during early gonadal development [39]. Nevertheless, wnt4 demonstrates sex dimorphic expression after gonadal differentiation and is selectively expressed in the ovary in nonmammalian vertebrates (e.g., chicken [13] and turtle [14]), including black porgy.

In the present studies, ovarian development occurred following the regression of testicular tissue in fish treated with E2 and AI. Immunohistochemical staining for Cyp19a1a [9] and Wnt4 (the present studies) showed that the expression of both was stronger in follicle cells surrounding female germ cells in E2-treated fish than in AI-treated fish. However, Wnt4-immunopositive cells were only observed in follicle cells around oogonia. In addition, wnt4 (in the present studies) and foxl2 [9] transcripts were higher in E2-stimulated ovarian tissue compared with AI-stimulated ovarian tissue. Aromatase inhibitor treatment induced ovarian growth and an increase in wnt4 transcripts but not foxl2 transcripts [9] in ≥0-yr-old and ≥1-yr-old black porgy. The expression of wnt4 was closely related to histological observations such as the proliferation of oogonia, early primary oocytes, and early growth of ovarian lamellae. Wnt4/Wnt4 expression was decreased, however, when the ovary entered the stage of late primary oocytes. Considering these data together, we suggest that wnt4 could be more important than Cyp19a1a (E2) for early ovarian development, especially oogonia proliferation. Wnt4 is also highly expressed in small growing follicles in the ovaries of immature mice [39]. Moreover, a recent study [40] showed that Wnt4 blocked androgen production by suppressing masculine gene expression (e.g., cyp11a, cyp17, hsd3b1, and hsd17b1), and hsd17b3) in female mice. The role of wnt4 in the ovary, however, appears to be more complicated in black porgy. The
expression of wnt4 was high in developing ovarian tissue during the nonspawning season (May), after the regression of testicular tissue in ≥1-yr-old and ≥2-yr-old fish. In addition, high expression of wnt4 was only observed in the active ovary (early vitellogenic oocytes [i.e., from primary oocyte to vitellogenic oocyte stage]) when the testis had regressed and the fish was proceeding to the natural sex change among ≥2-yr-old fish. In male mice, Fgf9 regulates Sox9 (a testicular gene) expression through the inhibition of wnt4 (an ovarian gene) in gonad development [41]. Thus, our data suggest that wnt4 expression in ovarian tissue is negatively regulated by factors released by testicular tissue.

To evaluate this hypothesis, we used a surgical operation to remove testicular tissue in ≥1-yr-old fish. Three months after testicular removal, ovarian tissue further developed, and the primary oocyte diameter increased. This surgical operation induced an early sex change in ≥1-yr-old black porgy similar to the natural sex change in ≥2- to 3-yr-old fish. Moreover, wnt4 was the first differentially expressed gene in testis-removed ovary 3 mo after testicular removal even when the expressions of cyp19a1a and other genes were unchanged. In addition, Pena staining data demonstrated that treatment with AI in ≥1-yr-old fish resulted in significant regression of testicular tissue [9] and increased wnt4 transcripts in ovarian tissue compared with control fish. Therefore, our data support the hypothesis that wnt4 expression may be suppressed to some extent by testicular factors. Even more striking, wnt4 was elevated in AI-treated ovarian tissue with low gonadal E2 levels, although short-term and long-term administration of E2 could stimulate an increase in wnt4 expression. This suggests that wnt4 expression could be regulated by factors other than E2.

In the natural sex change, wnt4 expression gradually increased after September (testicular tissue was already completely regressed) in the present study, while plasma E2 levels gradually increased in ≥2- to 3-yr-old fish in previous investigations [1]. Exogenous E2 could also stimulate an increase in wnt4 transcription in a dose-dependent manner in ≥2-yr-old fish. A novel role of estrogen involves its targeting of early Wnt4/beta-catenin signaling in an estrogen receptor-independent manner to regulate late uterine growth responses in mice [42]. In addition, plasma luteinizing hormone levels are markedly increased in sex changing black porgy [43]. Wnt4/ Fzd4 expression is stimulated by human chorionic gonadotropin or prolactin by secreting frizzled-related protein (SFRP4) in corpora lutea of mice [44]. The increase in wnt4 transcripts during the natural sex change may be partially due to luteinizing hormone and endogenous E2 stimulation, as elevated plasma levels of luteinizing hormone and E2 are detected in sex changing fish [1, 43].

In summary, the present studies demonstrate for the first time to date the possible roles of Wnt4/wnt4 throughout gonad development in a teleost model, the protandrous black porgy, including ovarian and testicular differentiation (≥0-yr-old fish), development of a bisexual gonad (≥0-yr-old and ≥1-yr-old fish), growth of ovarian lamellae (≥1-yr-old fish), and partial mediation of a later natural sex change (≥2-yr-old fish). Testicular factors could have negative effects on the sex change and the expression of ovarian wnt4. Our data demonstrate the involvement and association of wnt4 in early ovarian development and ovarian growth and maturation during the natural sex change but not in sex differentiation.

ACKNOWLEDGMENT

We thank American Journal Experts for editing.

REFERENCES


