BIOLOGY OF REPRODUCTION **79,** 1111–1120 (2008)
Published online before print 30 July 2008.
DOI 10.1095/biolreprod.108.069146

Dual Roles of *cyp19a1a* in Gonadal Sex Differentiation and Development in the Protandrous Black Porgy, *Acanthopagrus schlegeli*[1]

Guan-Chung Wu,[3] Sherly Tomy,[4] Masaru Nakamura,[5] and Ching-Fong Chang[2,3,4]

Institute of Bioscience and Biotechnology[3] and Department of Aquaculture,[4] National Taiwan Ocean University, Keelung 202, Taiwan
Sesoko Station,[5] Tropical Biosphere Research Center, University of the Ryukyus, Motobu 905-0277, Japan

ABSTRACT

Protandrous black porgy fish, *Acanthopagrus schlegeli,* have a striking life cycle, with male sex differentiation at the juvenile stage, a bisexual gonad during first 2 yr of life, and a male-to-female sex change (with vitellogenic oocytes) at 3 yr of age. The present study investigated the role of aromatase (*cyp19a1a*/Cyp19a1a) in gonadal development in this species, especially in relation to sexual differentiation and sex change. Fish of various ages were treated with estradiol (E_2) or aromatase inhibitor (AI) to determine whether manipulation of the hormonal environment has an impact on these processes. We report an integrative immunohistochemical, cellular, and molecular data set describing these interesting phenomena. During male sex differentiation, high levels of *cyp19a1a*/Cyp19a1a expression were observed in the undifferentiated gonad (4 mo of age), in marked contrast to the low *cyp19a1a*/Cyp19a1a levels detected in the differentiated testis at the age of 5–6 mo. A low dose of E_2 (0.25 mg/kg feed) stimulated testicular growth and function in sexually differentiated fish, whereas a high dose of E_2 (6 mg/kg feed) induced female development. Furthermore, administration of AI suppressed male development and promoted female sexual differentiation. An increased number of *figla* transcripts (an oocyte-specific gene) were observed prior to *cyp19a1a* expression, concomitant with the development of oogonia and early primary oocytes in the ovaries of both E_2- and AI-treated groups. Immunohistochemical Pcna staining showed that the regression of testicular tissue occurred prior to the development of ovarian tissue in both E_2- and AI-induced females. The importance of *cyp19a1a* in female development was further demonstrated by the increase in *cyp19a1a* transcripts during the naturally occurring sex change. Transcripts of *foxl2* increased in the gonads of 2- to 3-yr-old black porgy during the early stages of the natural sex change, followed by a gradual elevation of *cyp19a1a* levels. The levels of both genes peaked in the resulting ovarian tissue. Thus, *cyp19a1a*/Cyp19a1a plays dual roles in the gonadal development, namely, in testicular development during the initial period of sexual differentiation and later in ovarian development during the natural sex change.

early development, estradiol, male sexual function, ovary, testis

INTRODUCTION

Black porgy, *Acanthopagrus schlegeli* Bleeker, are marine protandrous hermaphrodite fish that possess 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 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 initial sex differentiation (5 mo of age) and the first reproductive season [3]. During the postspawning (April to May) and nonspawning (June to August) periods following the first reproductive cycle, the testicular tissue regresses, and the ovarian tissue expands to become the dominant tissue 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), 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% of the population. This subset will subsequently change sex by developing vitellogenic oocytes in the approaching third reproductive season [3, 4].

Plasma estradiol-17β (E_2) levels during 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]. However, plasma E_2 is maintained at low levels when ovarian tissue develops to become the dominant tissue during the postspawning and nonspawning seasons in 1^+- and 2^+-yr-old fish [1, 2, 4]. Furthermore, ovarian tissue is arrested at the primary oocyte stage until the sex change [5–7]. In our recent study, *cyp19a1a* transcript levels were high before male sex differentiation and dramatically decreased after the testis underwent early spermatogenesis [8]. Therefore, the possible role of *cyp19a1a* in the development of testicular tissue in addition to ovarian development deserves further study.

The gene *foxl2* has been reported to be related to *cyp19a1a* (aromatase, conversion of testosterone to E_2) and ovarian development [9]. *Foxl2* induces expression of downstream genes, including *Cyp19a1,* and has been observed to exhibit sexually dimorphic expression and be essential for the differentiation of granulosa cells in mouse ovaries [10]. The absence of *Foxl2* results in the blockage of granulosa cell differentiation and lack of secondary follicles formed after birth in mouse [10]. *Foxl2* is predominantly expressed in the ovary during and after female differentiation in nonmammalian vertebrates, including chicken [11], rainbow trout [12], and medaka [13]. *foxl2* upregulates *cyp19a1a* expression in vitro in tilapia [14]. The profile of *foxl2* expression in relation to gonad development is not yet clear in protandrous species.

[1]Supported in part by the National Science Council (Taiwan).
[2]Correspondence: FAX: 886 2 2462 1579;
e-mail: B0044@mail.ntou.edu.tw

Received: 9 March 2008.
First decision: 31 March 2008.
Accepted: 17 July 2008.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Specific primers used for quantitative real-time PCR analyses.

Gene	Orientation	Sequence
foxl2	Sense	5′-GAATAAAAAAGGCTGGCAGAACA-3′
	Antisense	5′-CCCGCGGAACTTTGATGA-3′
cyp19a1a	Sense	5′-ACAAACCCGACGAATTCAGACT-3′
	Antisense	5′-CCCGAACGGCTGGAAGTA-3′
nr5a4	Sense	5′-CAGCAAGTGGACTATGCAGTGAT-3′
	Antisense	5′-CTCCTGGGCATGGCTCAA-3′
figla	Sense	5′-CAGGAACTTGAACACCATGTTCTC-3′
	Antisense	5′-CTTACGGTCTGGTCGCATTAGTG-3′
gapdh	Sense	5′-AGGCTTCCTTAATCTCAGCATAAGAT-3′
	Antisense	5′-GGTGCCTGTGGCTGATGTG-3′

In the present study, we propose that Cyp19a1a (*cyp19a1a*) plays important roles in both testicular and ovarian development. For the present study, protandrous black porgy exhibiting the unique development of bisexual gonads and natural sex change were chosen for analysis of 1) *cyp19a1a* in relation to male sex differentiation, that is, from an undifferentiated stage to a fully developed male in juvenile (0^+-yr-old) fish, because of the mono-male sex pattern observed during sex differentiation; 2) Cyp19a1a/E_2 functions during sex differentiation and gonad development using exogenous E_2 and AI treatments of 0^+-yr-old fish; and 3) *foxl2* and *cyp19a1a* expression in relation to the natural sex change from male to female (development of advanced ovary, vitellogenic oocytes) in 2- to 3-yr-old fish.

MATERIALS AND METHODS

Experimental Fish

Three batches of black porgy, *Acanthropagus schlegeli*, were obtained for the experiments. Two-month-old fingerlings (0^+-yr-old fish, n = 270, body weight [BW]: 1.53 ± 0.20 g, body length: 4.62 ± 0.17 cm) were obtained from a pond culture in April for examination of sexual differentiation and for the experiments involving E_2 and AI administration. Six-month-old black porgy (n = 60, BW: 75.9 ± 4.3 g) were obtained in September to study the effects of various doses of E_2 on gonadal development. Two-year-old black porgy (n = 40, BW: 363.93 ± 18.19 g, gonadosomatic index [GSI]: 1.04 ± 0.19) were obtained in February to study the natural sex change and gene profiles during the annual reproductive cycle. Total body and gonadal weight were measured for the calculation of GSI (GSI = gonadal weight/body weight × 100).

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

Experimental Design

Experiment 1: morphological changes and gene profiles during male sex differentiation and E_2-induced female sex differentiation in 0^+-yr-old fish. Sexually undifferentiated juveniles were obtained to examine the expression profiles and morphological changes in gonads during sex differentiation and to determine the effect of E_2 administration. Fish (3 mo old) were divided equally into two groups—control and E_2 treated—in June 2005. The E_2 (Sigma, St. Louis, MO) was dissolved in ethanol and mixed with feed and water to obtain a final concentration of 6 mg per kg feed and was administered to the treatment group from June to January of the following year (7 mo). The control group was fed with feed devoid of E_2. Fish gonads were collected from June (3 mo old, undifferentiated gonad) to January (10 mo old, differentiated gonad) for expression analyses (n = 8 fish per group), histology, and immunohistochemical staining with Cyp19a1a antiserum (n = 6 fish per group). Due to the difficulty of collecting blood samples from small fish (3- to 5-mo-old fish), E_2 measurements were performed only in 6-mo-old fish.

Experiment 2: morphological changes and gene profiles of AI-induced females in 0^+-yr-old fish. In order to further clarify the importance of *cyp19a1a*/Cyp19a1a for testicular differentiation, AI treatments of undifferentiated fish were conducted. Fish (3 mo old) were divided equally into two groups, control and AI treated. The AI (1,4,6-androstatrien-3,17-dione; Steraloids Inc., Newport, RI) was dissolved in ethanol and mixed with feed and water to obtain a final concentration of 20 mg AI per kg feed and was administered to the treatment group from June to January of the following year (7 mo). Control fish were fed with a diet devoid of AI. Fish gonads (n = 8 fish) were collected monthly from July (4 mo old, undifferentiated gonad) to January (10 mo old, differentiated gonad) for gene analyses (n = 8 fish per group), histology, and immunohistochemical staining with Cyp19a1a antiserum (n = 6 fish per group). Due to the problems encountered in collecting blood samples from small fish (3- to 5-mo-old), E_2 measurements were only performed for 6-mo-old fish.

Experiment 3: gonadal development in 0^+-yr-old differentiated fish treated with various doses of E_2. In order to examine the effects of E_2 on testicular/ovarian development in sexually differentiated fish, 7-mo-old differentiated black porgy were divided into three groups (n = 20 per group)—control, low E_2 (0.25 mg/kg feed), and high E_2 (6.0 mg/kg feed)—in September 1991. Gonads and blood samples from 1-yr-old fish were collected 6 mo after E_2 administration (in February, during the first spawning season) to investigate gonadal status as well as plasma 11-ketotestosterone and E_2 levels.

Experiment 4: gene profiles during the natural sex change in 2- to 3-yr-old fish. In order to examine the expression profiles in gonads during the natural sex change, 2-yr-old black porgy were collected regularly (n = 8 fish) at 3-mo intervals from January 2002 (second spawning) to January 2003 (after sex change). Ovarian samples were collected to examine the status of sex (male or female) and for gene analyses.

Genes Studied Based on Known Roles in Gonad Development

The expression profiles of genes known to be involved in gonadal differentiation and development in other vertebrates, including *cyp19a1a*, *nr5a1a*, *figla,* and *pcna*, were examined in this study. *cyp19a1a* encodes an important steroidogenic enzyme that converts testosterone to estrogen and is known to play a role in ovarian tissue [8]. Steroidogenic factor *nr5a1a* (previously named *nr5a4*) has been demonstrated to be an important factor for male sex differentiation in black porgy [8]. Also monitored in relation to gonad development were factor in germline alpha (*figla*), as an oocyte-specific marker and inducer of folliculogenesis [15], and proliferating cell nuclear antigen (*pcna*), expressed in G_1/S phase of the cell cycle, as an indicator of the proliferation activities in the cell/tissue [16].

Quantification of foxl2, cyp19a1a, figla, *and* nr5a1a *Transcripts by Quantitative Real-Time PCR Analysis*

Quantitative real-time PCR analysis of gene transcripts of *foxl2* (ACA97597), *cyp19a1a* (*P450arom*, AY870246), *figla* (ACA97598), *nr5a1a* (AY491379), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh,* DQ399798) were conducted according to the methods described in previous studies [8, 17]. Specific primers were designed for quantitative real-time PCR (Table 1). 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 and Immunohistochemical Staining with Cyp19a1a and PCNA Antisera

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

For immunohistochemical staining, the section was rehydrated in a sodium phosphate buffer with saline (PBS) and incubated with 3% H_2O_2 in PBS. The section then was incubated with 1.5% normal goat serum for 30 min and with antiserum against human PCNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or antiserum against aneomonefish (*Amphiprion clarkia*) Cyp19a1a (N-terminal peptide) overnight at 4°C. This was followed by incubation with

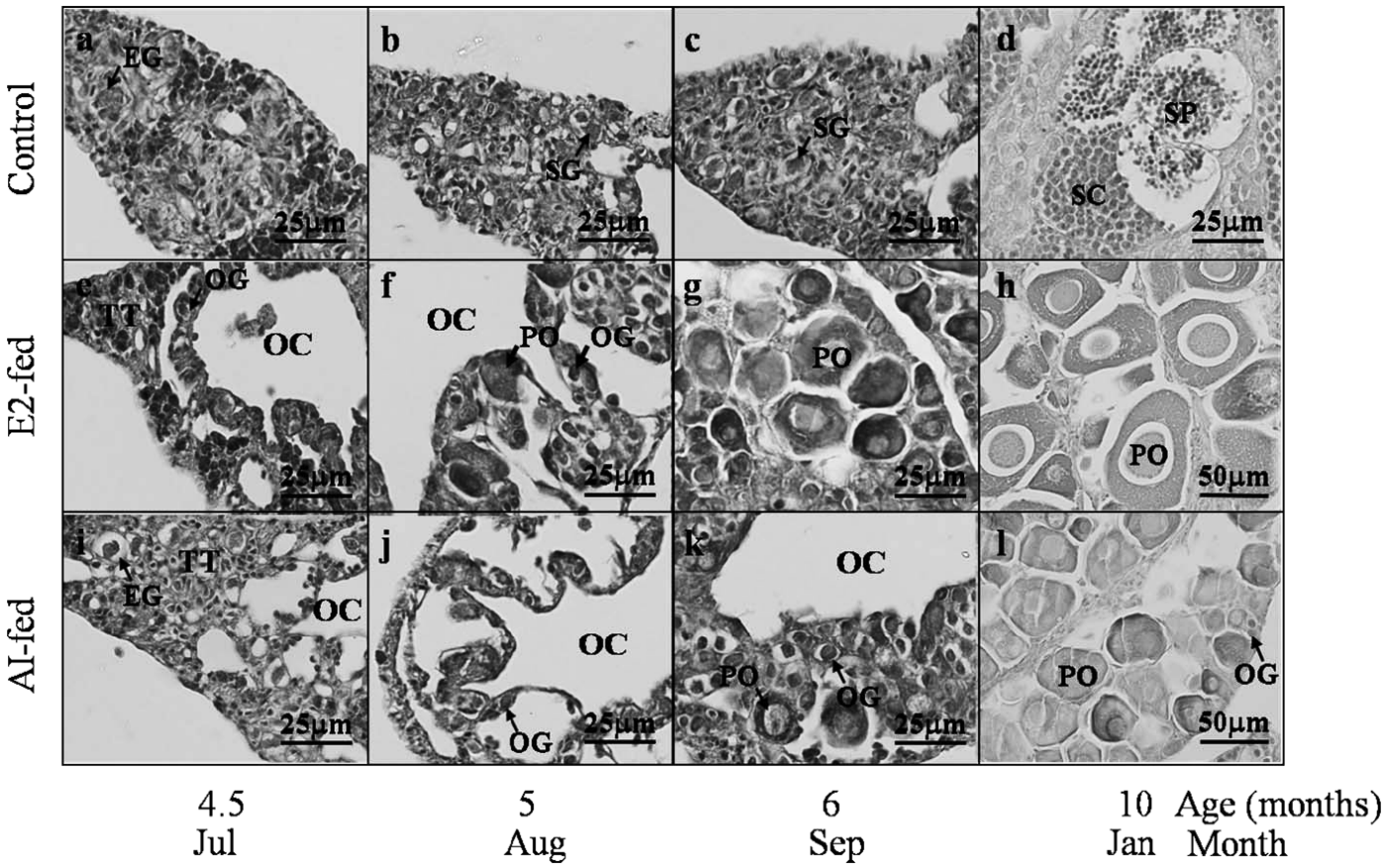

FIG. 1. Effects of E_2 and AI on the gonadal development of black porgy. The E_2 (6 mg/kg feed) or AI (20 mg/kg feed) was fed to fish from ages 3 to 10 mo (June 2005 to January 2006). Transverse sections of gonad tissue stained with hematoxylin-eosin in the control (**a–d**), E_2-fed (**e–h**) and AI-fed (**i–l**) fish are shown. EG, Early germ cell; OC, ovarian cavity; OG, oogonia; PO, primary oocyte; SG, spermatogonia; SC, spermatocyte; SP, spermatid; TT, testicular tissue.

biotinylated anti-rabbit IgG (H + L) from Vector Laboratories Inc. (Burlingame, CA). Color formation was amplified with an ABC kit (avidin-biotin; Vector Laboratories) and 3,3′-diaminobenzidine (Sigma). The PCNA antiserum was induced in the rabbit against human PCNA, which has 92% amino acid identity with the black porgy. The Cyp19a1a antiserum was induced in rabbit against an anemonefish Cyp19a1a peptide fragment (CNLSQQPVEHQQEAERL). The 14 underlined amino acids of this peptide fragment had 100% identity with the black porgy protein.

Radioimmunoassay of Plasma 11-Ketotestosterone and E_2 Levels

Plasma 11-ketotestosterone and E_2 levels were measured with a validated radioimmunoassay technique. Radioimmunoassay was performed following ethyl ether extraction, as described previously [2]. The radioactivity of the bound ^{3}H-steroid in the supernatant fluid was counted in a liquid scintillation spectrophotometer (Beckman 1801) after being mixed with a scintillation cocktail (Ready Safe; Beckman Inc., Fullerton, CA). The [2,4,6,7-^{3}H]-estradiol-17β (99 Ci/mmol) was purchased from NEN Research Products (Boston, MA). The [1,2-^{3}H]-11-ketotestosterone (51.9 Ci/mmol; Amersham Co., Arlington Heights, IL) was a gift from Dr. P. Thomas (University of Texas, Austin, TX). Intraassay and interassay variations ranged between 11.5% and 17.0%. Cross-reaction of the antisera against E_2 and 11-ketotestosterone was evaluated as described previously [18, 19].

Data Analysis

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

RESULTS

Morphological Changes and Gene Profiles During Male Sex Differentiation and E_2-Induced Female Sex Differentiation in 0^+-Yr-Old Fish

Morphological changes. In the control fish, undifferentiated gonad tissue containing early-stage germ cells was observed in 4.5-mo-old fish (July 2005; Fig. 1a). Spermatogonia appeared in August (at 5 mo; Fig. 1b). Lobular testicular tissue with late spermatogonia and spermatocytes appeared in September (at 6 mo; Fig. 1c). Active spermatogenesis (yielding spermatocytes and sperm) was observed in January 2005 (at 10 mo; Fig. 1d). Ovarian tissue formed a very small portion of the bisexual gonad in 0^+- to 1-yr-old fish, with only a few primary oocytes present in the ovarian cavity (data not shown).

After 1.5 mo of E_2 administration (at 4.5 mo of age, in July), ovarian tissue with few oogonia was observed together with the regressed testicular tissue (Fig. 1e). Many primary oocytes were found in the ovarian tissue of E_2-treated fish at 5 and 6 mo of age, in August and September 2005 (Fig. 1, f and g), and the testicular tissue appeared to have completely regressed (Fig. 1, g and h).

Plasma E_2 levels. After 3 mo of E_2 administration, plasma E_2 concentrations in 6-mo-old fish were 58.8 ± 3.5 pg/ml and 154.0 ± 12.8 pg/ml ($P < 0.05$) in the control and E_2 groups, respectively. Thus, E_2 administration resulted in high levels of plasma E_2 and induced female sexual development.

TABLE 2. The effects of various doses of E2 on the gonadal development and sex steroid levels in differentiated 6-month-old black porgy.

Group	GSI	11-KT (ng/ml)*	E2 (pg/ml)	Gonadal stage
Control	0.25 ± 0.01[b]	0.25 ± 0.05[b]	43.75 ± 12.5[a]	Mature testes
E2 (0.25 mg/kg of feed)	3.22 ± 0.74[a]	2.20 ± 0.50[a]	68.75 ± 15.0[a]	Mature testes
E2 (6 mg/kg of feed)	0.20 ± 0.05[b]	0.02 ± 0.00[c]	84.13 ± 31.3[a]	Ovary (primary oocyte)

* 11-KT, 11-ketotestosterone.
[a,b,c] Different superscript letters indicate significant differences ($P < 0.05$) between groups.

Gene profiles. In the control fish, *nr5a1a* expression was low in the undifferentiated gonads of 4- and 4.5-mo-old fish, but it increased in the differentiated males (1.8-fold in 5-mo-old fish and 2.8-fold in 6-mo-old fish; Fig. 2a). *foxl2* exhibited no consistent change in expression in control fish, but the levels were generally low (Fig. 2b). In contrast, *cyp19a1a* expression levels were lower in the differentiated gonad (5- or 6-mo-old fish) than in the undifferentiated gonad (4- or 4.5-mo-old fish; Fig. 2c). *figla* exhibited a gradual increase over time in the control gonads, yet it remained relatively low (Fig. 2d).

In the E_2-administered fish, the expression of *nr5a1a* dramatically decreased and was maintained at low levels in E_2-induced females compared with the control fish (2.8- to 14.6-fold decrease; Fig. 2a). The *foxl2* transcripts were low in the gonad at 4 and 4.5 mo of age and then increased in 5- and 6-mo-old fish (Fig. 2b). Estradiol induced an increase in *foxl2* transcripts in 5-mo-old (1.7-fold increase) and 6-mo-old (2.3-fold increase) fish but not in 4- and 4.5-mo-old fish compared with the controls (Fig. 2b). Lower expression of *cyp19a1a* at 4 (0.27-fold) and 4.5 (0.17-fold) mo of age and higher expression at 5 (1.2-fold) and 6 (3.2-fold) mo of age were found in the E_2-treated fish compared with the control fish (Fig. 2c). *figla* expression increased significantly in the E_2-induced females compared with the control males (104-fold, 565-fold, and 458-fold, respectively at 4.5, 5, and 6 mo of age; Fig. 2d).

Treatment with AI Resulted in Female Sex Differentiation

Morphological changes. Oral administration of AI induced the formation of an ovarian cavity at 4.5 mo of age (Fig. 1g) and further promoted the development of ovarian tissue into a structure of epithelial lamellae, together with the regression of testicular tissue, at 5 mo of age (Fig. 1j). Primary oocytes, in addition to oogonia, appeared at 6 and 10 mo of age (Fig. 1, k and l). The GSIs in the AI and control groups at 10 mo of age were 0.07 ± 0.01 and 0.06 ± 0.01 ($P > 0.05$), respectively.

Plasma E_2 levels. After 3 mo of AI administration in 6-mo-old fish, plasma E_2 concentrations were 58.8 ± 3.5 pg/ml and 40.0 ± 4.0 pg/ml ($P < 0.05$) in the control and AI groups, respectively.

Gene profiles. Low, constant levels of *nr5a1a* were found in the AI-induced female gonad compared with the increasing levels seen in the control (differentiated male gonad; Fig. 2e). The AI treatment resulted in the decrease of *foxl2* and *cyp19a1a* expression in 4- and 4.5-mo-old-fish (Fig. 2, f and g). Increased levels of *foxl2* and *cyp19a1a* expression were found in the gonad at 5 and 6 mo of age (active development of ovarian tissue) compared with the gonad at 4 and 4.5 mo of age (less active stage) in the AI group (Fig. 2, f and g). Aromatase inhibitor caused a significant increase ($P < 0.05$) in *cyp19a1a* but not *foxl2* expression in 6-mo-old fish compared with the control fish (Fig. 2, f and g). *figla* expression increased significantly in the gonads of 5- and 6-mo-old fish compared with the control group (Fig. 2h).

Comparison of Oocyte Diameter, Cellular Activities, and Cyp19a1a Expression in AI- and E_2-Induced Female Gonads

Oocyte diameter. The GSIs in AI-induced females (0.07 ± 0.01) and control males (0.06 ± 0.01) were smaller ($P < 0.05$) than in the E_2-induced group (0.16 ± 0.02) at 10 mo of age. The oocyte diameters were significantly smaller in the AI-administered group compared with the E_2 group (Fig. 3).

Cellular activities. In the control fish, the immunohistochemical staining for Pcna (a marker for proliferative activity of cells) revealed that the undifferentiated gonad (4.5 mo of age) contained only a few somatic cells (putative Sertoli cells, according to cell morphology) with positive Pcna staining, and a population of early germ cells with no Pcna staining (Fig. 4a). In the differentiated gonad (5 and 6 mo of age; Fig. 4, b and c), an increase in the number of both somatic cells (putative Sertoli cells) and spermatogonia with *pcna*-positive staining was observed.

In the E_2-treated fish, gonads had few Pcna-positive oogonia in the ovarian tissue at 4.5 mo of age (Fig. 4d). More actively proliferating ovarian tissue was observed in the E_2-treated gonads of 5- and 6-mo-old fish (Fig. 4, e and f). Active primary oocytes also were observed in the E_2-treated gonads at 6 mo of age (Fig. 4f). Regressed testicular tissue with no positive Pcna staining was found in both AI- and E_2-induced gonads (Fig. 4, d, e, g, and h). Primary oocytes were observed at 6 mo (Fig. 4, f and i) and 10 mo (Fig. 1, h and l) of age in both AI and E_2 groups.

Cyp19a1a immunohistochemical staining. In the controls, stronger Cyp19a1a staining was found in the undifferentiated gonad (Fig. 5, a and b) compared with the differentiated gonad (Fig. 5c). Based on the immunohistochemical staining, the Cyp19a1a protein was expressed in the follicle cells around the germ cells (oogonia and primary oocytes), but more faintly in the AI females (Fig. 5, j–o) compared with the E_2 group at various ages (4.5–6 mo of age; Fig. 5, d–i).

Various Doses of E_2 Administrated to Differentiated Males Resulted in the Development of Testicular or Ovarian Tissue

To further investigate the effects of E_2 on testicular development, various doses of E_2 were administered to differentiated males (7-mo-old to 1-yr-old fish) for 6 mo (from September to February of the next year). A high dose of E_2 (6 mg/kg feed) induced female development (ovary, primary oocytes; Table 2). In contrast, a low dose of E_2 (0.25 mg/kg feed) induced testicular development with a high GSI (active and functional testis), together with increased plasma 11-ketotestosterone concentrations compared with the control males (Table 2). No difference in the levels of plasma E_2 was found between the control and E_2-treated groups (Table 2).

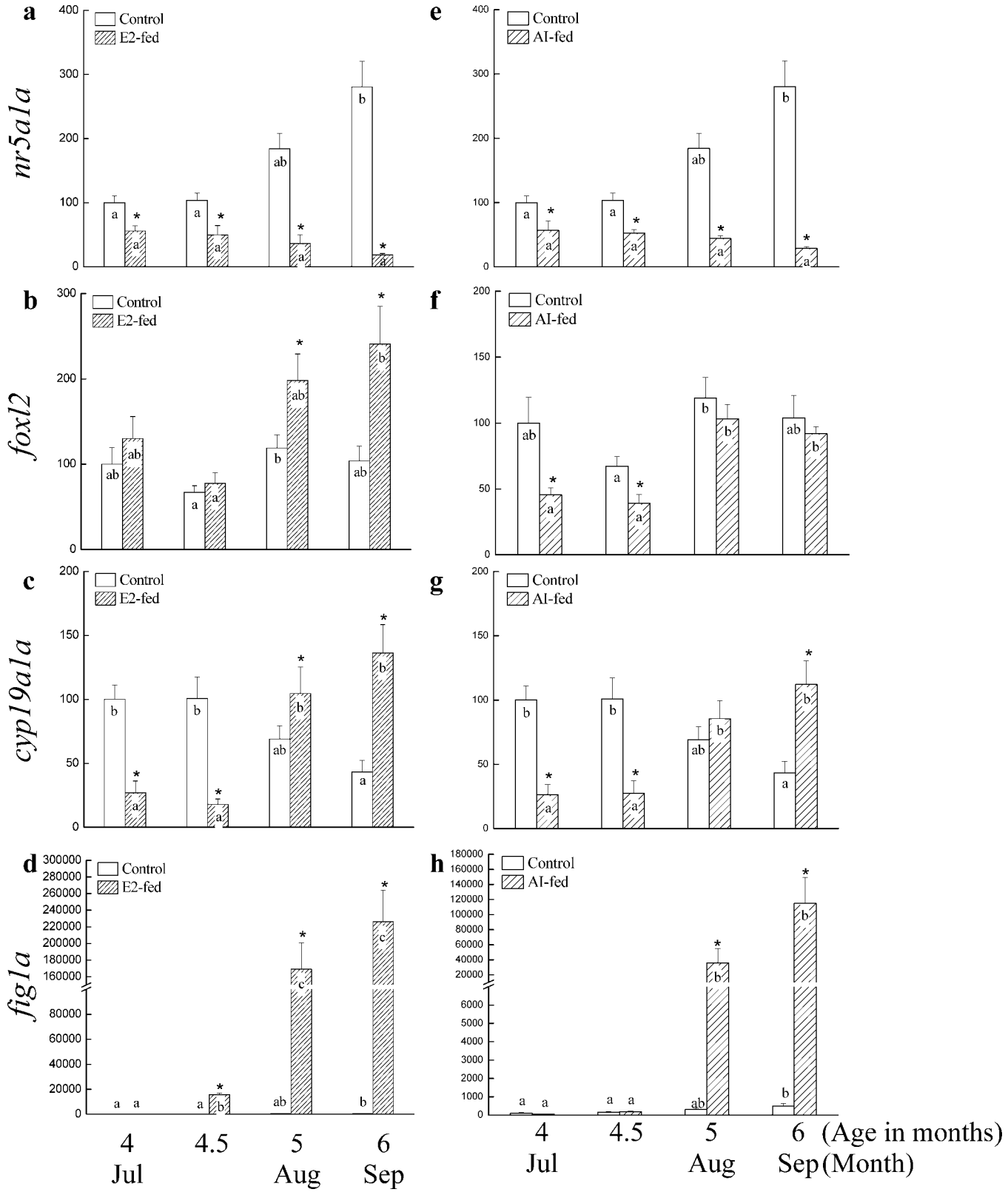

FIG. 2. Relative gene transcripts of *nr5a1a* (**a**, **e**), *foxl2* (**b**, **f**), *cyp19a1a* (**c**, **g**), and *figla* (**d**, **h**) in the gonad tissue of juvenile black porgy in the control vs. E_2-fed groups (**a**–**d**) and control vs. AI-fed groups (**e**–**h**). Testicular tissue was the main tissue in the control gonad for gene analysis. Administration of E_2 (6 mg/kg feed) and AI (20 mg/kg feed) was conducted for a period of 7 mo. The values were calibrated with the internal control, *gapdh,* with the value at 4 mo of age defined as 100%. Each value was expressed as mean ± SEM. Different letters represent significant differences ($P < 0.05$) in gene transcripts at various ages in the same group (n = 8 in each value). *Significant difference ($P < 0.05$) in the control vs. the E_2-fed fish and control vs. the AI-fed fish.

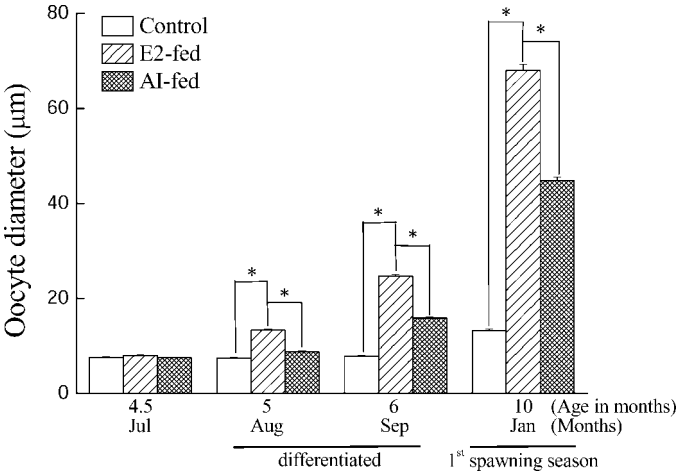

FIG. 3. The development of germ cells in the control, E_2-fed (6 mg/kg feed), and AI-fed (20 mg/kg feed) fish. The oocyte cell diameters were measured during the period of differentiating sex (4.5 mo of age), differentiated sex, and at the beginning of the first spawning season (5–10 mo of age). *Significant difference ($P < 0.05$) between the control and AI-fed or E_2-fed groups.

Ovarian foxl2 *and* cyp19a1a *Expression Profiles in 2+- to 3-Yr-Old Fish During Natural Sex Change*

The ovarian portion of the gonad increased in size significantly during the postspawning season, further developing into a full ovary during the third spawning season in naturally sex-changed fish (Fig. 6a).

Quantitative real-time PCR data showed that ovarian *foxl2* transcripts were low in the second spawning season, significantly increased during the prespawning season, and reached the highest levels at the third spawning season (Fig. 6b). Conversely, ovarian *cyp19a1a* expression remained low during the second spawning, postspawning, nonspawning, and prespawning seasons, even though the ovarian tissue increased in proportion to the rest of the gonad (Fig. 6c). Ovarian *cyp19a1a* transcripts significantly increased in the female during the third spawning season (Fig. 6c).

DISCUSSION

We report here, for the first time, histological, cellular, and molecular data on testicular differentiation and ovarian development in relation to the roles of *cyp19a1a*/Cyp19a1a in a nonmammalian model organism, protandrous black porgy fish. The mono-male sex differentiation in this fish species provides a unique model to study the molecular mechanism of sex differentiation (at 5 mo of age) and later during the natural sex change (2–3 yr of age). The present data confirmed the previous findings that gonadal sex differentiation occurred at the age of 5 mo in black porgy, coincident with increased *nr5a1a* transcripts [8].

Transcript levels of *cyp19a1a* were significantly higher in undifferentiated gonads compared with newly differentiated gonads. Cyp19a1a protein expression was also stronger in the undifferentiated gonad, based on immunohistochemical staining with Cyp19a1a antiserum. The requirement and importance of Cyp19a1a for testicular differentiation were further demonstrated by long-term treatment with AI. Exogenous AI and E_2 administration resulted in decreased *cyp19a1a* transcripts in the first 1.5 mo of administration (before ovarian development, at 4 and 4.5 mo of age). Transcripts of gonadal *figla* (a female germ cell-specific gene) simultaneously increased after 1.5 mo of administration (at 4.5 mo of age) in the E_2 group and after 2 mo of administration (at 5 mo of age) in the AI group. Active proliferation of oogonia (based on Pcna immunohistochemical staining) and the development of primary oocytes occurred at the age of 4.5 mo in the E_2-administered group and 5 mo in the AI-administered group, when *cyp19a1a* transcripts were still low (compared with the control). In addition, AI treatment also resulted in lower levels of plasma E_2, as observed in 6-mo-old AI-induced females (about 40 pg/ml) compared with the control fish (about 58 pg/ml). These plasma E_2 concentrations in the control fish were

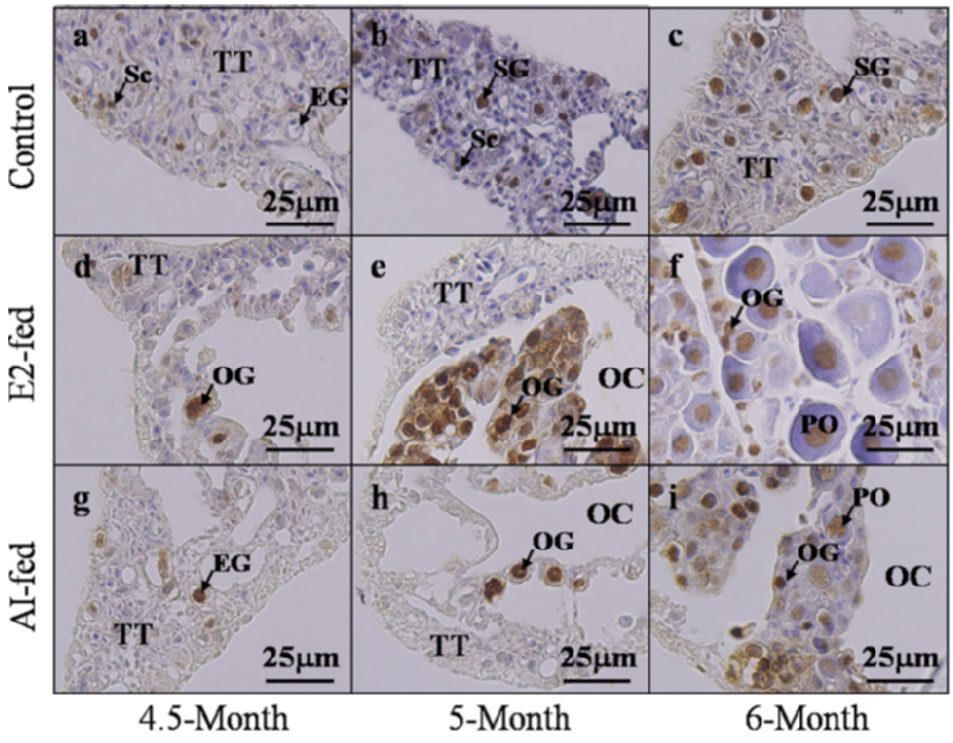

FIG. 4. Cellular activity during gonadal differentiation in the control, E_2-fed, and AI-fed fish. The E_2 (6 mg/kg feed) or the AI (20 mg/kg feed) was given to undifferentiated fish (from 3 mo of age). Transverse sections of gonadal tissue were immunohistochemically stained for Pcna (brown) and counterstained with hematoxylin (purple) in the gonad of control (**a**–**c**), E_2-fed (**d**–**f**), and AI-fed (**g**–**i**) fish. Pcna is expressed in the G_1/S phase of the cell cycle, providing evidence for cell cycling activity. EG, Early germ cell; OC, ovarian cavity; OG, oogonia; PO, primary oocyte; Sc, somatic cell (putative Sertoli cell); SG, spermatogonia; TT, testicular tissue.

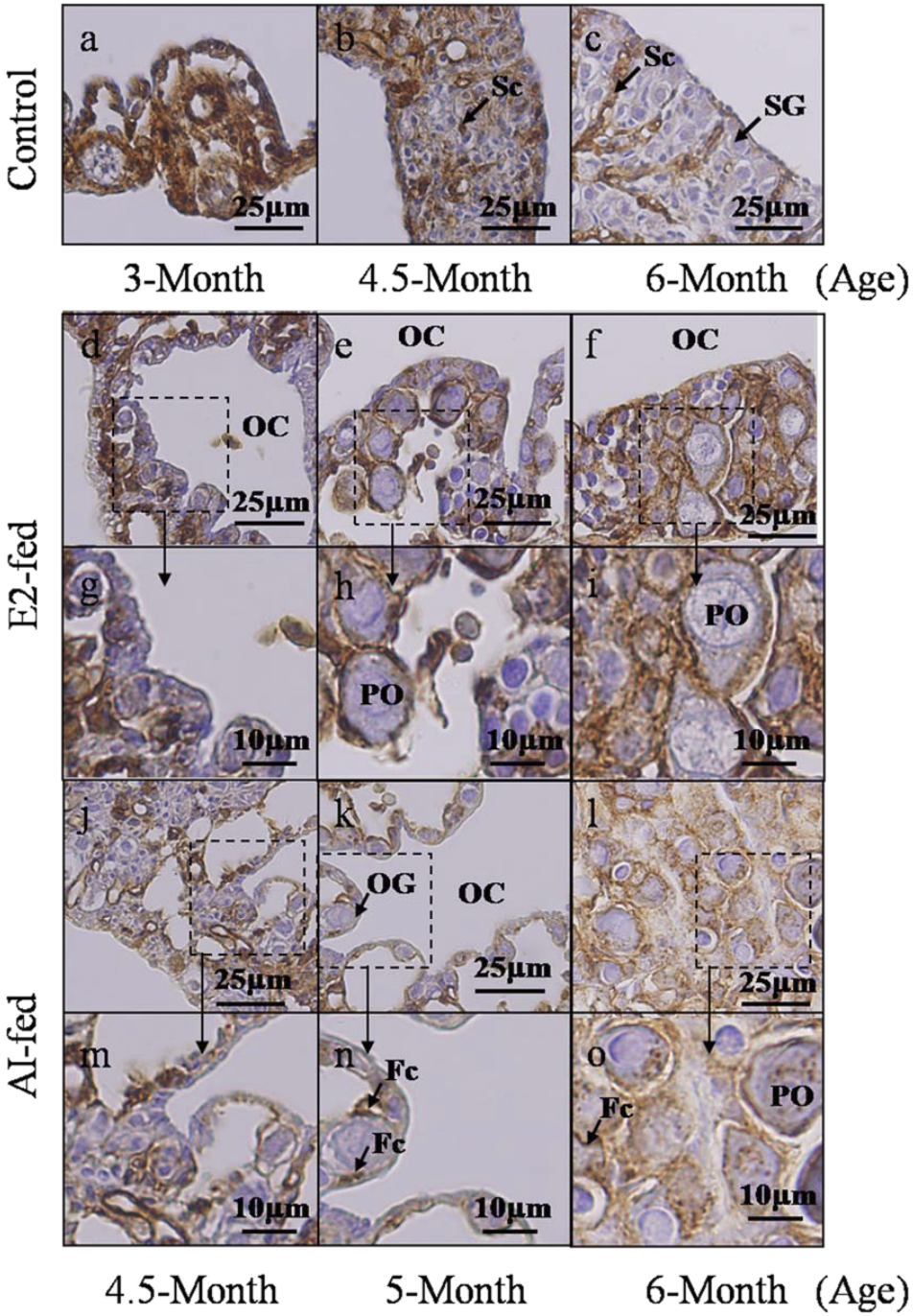

FIG. 5. Development of gonad during male sex differentiation in the control (**a**–**c**) and follicles (oocytes and follicle cells) in E_2-administered (**d**–**i**) and AI-administered (**j**–**o**) groups. The E_2 (6 mg/kg feed) or AI (20 mg/kg feed) was given to undifferentiated fish (3 mo of age). Transverse sections of gonadal tissue were immunohistochemically stained for Cyp19a1a (brown) and counterstained with hematoxylin-eosin (purple) in the controls (**a**–**c**), E_2-administered fish (**d**–**i**), and AI-administered fish (**j**–**o**). Cyp19a1a was expressed in follicle cells of the gonad. **g**, **h**, **i**, **m**, **n**, and **o** are the magnified images of **d**, **e**, **f**, **j**, **k**, and **l**, respectively. Fc, Follicle cell; OC, ovarian cavity; OG, oogonia; PO, primary oocyte.

very similar to our previous data collected for bisexual black porgy at the male phase with arresting primary oocytes [1, 2]. Plasma E_2 levels 2- to 3-fold higher than those of male fish might be significantly related to the stage of active oocyte growth. In the AI group, gonadal *cyp19a1a* transcripts were elevated at the age of 6 mo (active development of ovarian tissue) compared with fish at the age of 4 and 4.5 mo (less active stage) (Fig. 2). Therefore, AI could not suppress E_2 levels to a dramatically low concentration at this stage.

The data thus indicate that testicular differentiation but not early ovarian development is related to the level of *cyp19a1a* transcripts. The importance of E_2, the product of Cyp19a1a enzymatic activity, for testicular growth/development was further investigated by administering a low dose of E_2 (0.25 mg/kg feed) in the feed. Long-term, low-dose E_2 administration clearly enhanced testicular development, as revealed by the growth of a large and active testis and high levels of plasma 11-ketotestosterone (a key sex steroid closely associated with testicular function in fish). Previous studies showed that an increase of testosterone engendered by testosterone administration (4 mg/kg feed) resulted in testicular growth and male function in 1-yr-old black porgy [20]. Therefore, the possibility that a low ratio of E_2:testosterone initiates ovarian development in AI-treated fish is not supported in the present study. Taking all these data together, it is suggested that Cyp19a1a expression is important and necessary for testicular differentiation and development in the protandrous black porgy.

Estrogens have long been considered important for female sexual differentiation in noneutherian vertebrates. Administration of estrogen resulted in the conversion from male to

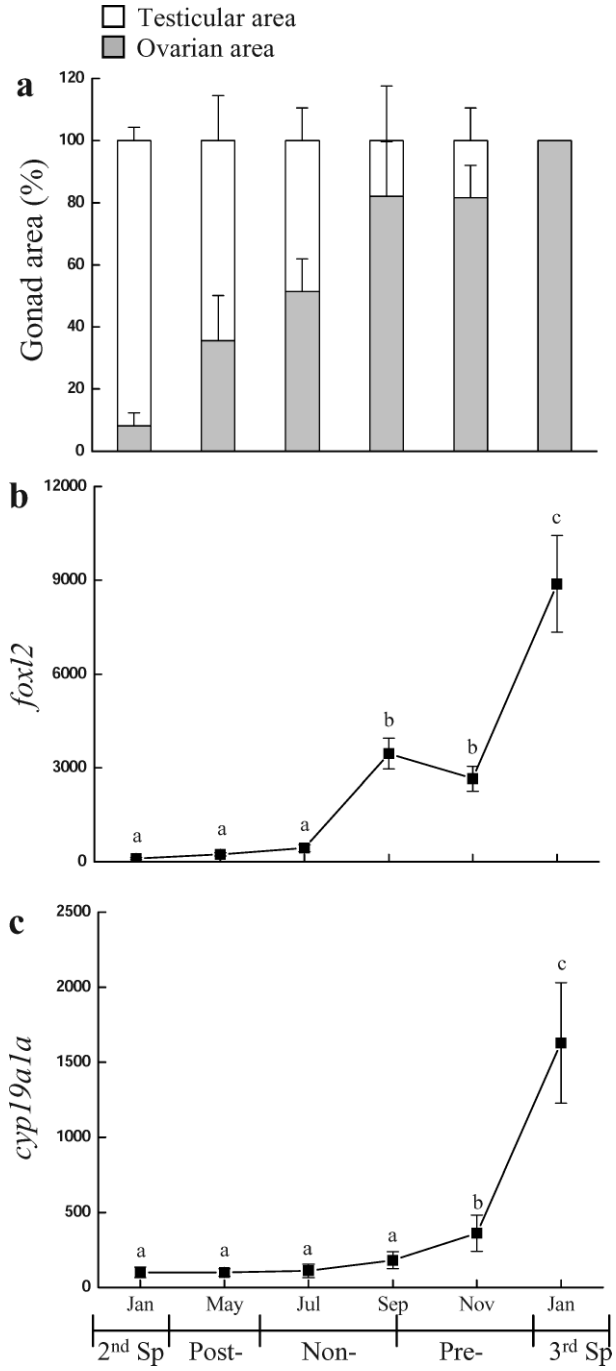

FIG. 6. Gonadal status and relative expression profiles of *foxl2* and *cyp19a1a* in ovarian tissue during the natural sex change from 2- to 3-yr-old fish. **a**) The ratio between ovarian area (gray parts of the bars) and the whole area of the gonad (full bar). The mRNA levels of *foxl2* (**b**) and *cyp19a1a* (**c**) were measured by real-time PCR in the ovarian tissue. The values were calibrated with the internal control, *gapdh,* with the value in January of the second spawning season defined as 100%. Each value was expressed as mean ± SEM. Different letters represent statistical differences ($P < 0.05$) among the groups (n = 8 in each value). Non-, Nonspawning season; Pre-, prespawning season; Post-, postspawning season; Sp, spawning season.

phenotypic female in marsupials [21], birds [22], reptiles [23], and teleosts [24, 25]. Treatment with AI to block Cyp19a1a action also resulted in the production of phenotypic males from females in birds [26, 27], reptiles [28, 29], and teleosts, such as tilapia [30], rainbow trout [30], and medaka [31]. Thus, endogenous estrogens are traditionally considered to be important for female sexual differentiation and development. In contrast, our present study clearly demonstrated that endogenous Cyp19a1a together with low levels of exogenous E_2 were important for male sexual differentiation and development. The data further suggest that Cyp19a1a had no significant impact on the early development of ovarian tissue/female germ cells. Our present findings were consistent with several published studies. Renewal of spermatogonia could be promoted by exogenous estrogens in Japanese eel (*Anguilla japonica*) [32] and Japanese huchen (*Hucho perryi*) [33]. Oogenesis and folliculogenesis were also not affected by AI in medaka [31]. Exogenous low E_2 (0.25 and 1.0 mg/kg feed) [6] and high testosterone (4 mg/kg feed) [20] stimulated testicular functions by enhancing spermiation and testis weight in black porgy; furthermore, low E_2 had stronger effects than high testosterone. Disruption of the *Cyp19a1a* gene in mice did not affect early ovarian development, but it did result in later ovarian regression at the postnatal stage [34].

On the other hand, our data also demonstrate the involvement of Cyp19a1a in ovarian development, judging by experiments involving E_2 and AI administration (experiments 1 and 2), and in the process of natural sex change (experiment 4). Exogenous E_2 (4–6 mg/kg feed) successfully induced sex change in 0^+- to 1-yr-old and 1^+- to 2-yr-old black porgy in the present and in previous studies [1, 8, 35]. Therefore, treatment with a certain amount of E_2 (the high dose in the present study) caused an increase in plasma E_2 levels and resulted in female development. Although AI administration also induced ovarian development, the diameters of primary oocytes in AI-induced females (experiment 2) were smaller than those in E_2-induced females (experiment 1). Additionally, the expression of Cyp19a1a protein in the ovarian tissue was faint in AI-induced females (experiment 2) compared with E2-induced females (experiment 1; Fig. 5). Weak expression of Cyp19a1a in the follicle cells surrounding the primary oocytes may be related to the development of small primary oocytes in the AI-induced group. The importance of *cyp19a1a* in the natural sex change was also demonstrated in the present study. Increased ovarian *cyp19a1a* transcripts were found in the late stages of the natural sex change in 3-yr-old fish. Furthermore, previous studies demonstrated that long-term administration of AI in 2^+-yr-old black porgy resulted in the blockage of the natural sex change at 3 yr of age [5]. Therefore, *cyp19a1a* must play an important role in the development of late primary oocytes and vitellogenic oocytes in black porgy.

The high dose of exogenous E_2 (6 mg/kg feed) and AI (20 mg/kg feed) both induced testicular regression within 1–2 mo of administration, according to the histological observations made in the present study. As immunohistochemical staining against Pcna proved to be an appropriate indicator for the status of the proliferative activities in the gonad, we were also able to determine that E_2- and AI-treated animals exhibited decreased levels of cell proliferation in the testicular tissue. Previous histological observations also indicated that testicular tissue regressed and the bisexual gonad became a full ovary (but still at the primary oocyte stage) during the early prespawning season (before sex change occurred) within the natural sex change process in 2- to 3-yr-old black porgy [3]. We also have observed that in order for ovarian development to occur (including sex change) in black porgy, the testicular tissue had to first become inactive/regressive (compared with control testicular tissue). The interaction between testicular and ovarian

tissue is thus an interesting and important aspect of sexual development.

We found that levels of *foxl2* transcripts increased earlier (September) than those of *cyp19a1a* (November) during the natural sex change (Fig. 6). The difference between E_2 and AI actions lay in their dissimilar effects on *foxl2* expression (Fig. 2). *foxl2* expression was not significantly affected by either E_2 or AI treatment at 4 or 4.5 mo of age, although levels were lower in the AI-treated group compared with the E_2-treated group. Administration of E_2, but not AI, induced high levels of ovarian *foxl2* transcripts at 5 and 6 mo of age. The E_2 treatment also resulted in higher expression of *cyp19a1a* in 5- and 6-mo-old fish compared with the controls; in contrast, the stimulatory effect of AI on *cyp19a1a* was observed only at 6 mo of age. Low expression of *foxl2* (in 4- to 5-mo-old fish) in the AI group compared with the E_2 group may have resulted in the decreased expression of Cyp19a1a protein in the follicle cells as well as the smaller size of primary oocytes. *Foxl2*/*foxl2* has been shown to positively regulate *cyp19a1a* expression in mammals [10] and fish [14]. Administration of E_2 also induced an increased *foxl2* expression and ovarian differentiation in rainbow trout [12] and medaka [13]. Treatment with AI decreased *FOXL2* levels in chicken [27]. *Foxl2* mutant mice displayed an ovarylike stroma with normal oocytes at the fetal stage, but testislike supporting cells were later formed in postnatal ovary (an incomplete sex reversal) [36]. Therefore, *foxl2* in combination with *cyp19a1a* may help to regulate the later development of ovarian tissue in black porgy, which includes processes such as the development of follicle cells and folliculogenesis.

In summary, the present study demonstrated the dual roles of *cyp19a1a* throughout gonad development in the protandrous black porgy, during which time both testicular and ovarian tissues differentiate within the bisexual gonad. The involvement and association of *cyp19a1a* in testicular development during early sexual differentiation as well as in ovarian development during the later natural sex change were conclusively determined. Testicular regression was shown to be important for the initiation of ovarian development in the bisexual gonad.

ACKNOWLEDGMENT

We thank American Journal Experts for the editing.

REFERENCES

1. Chang CF, Lee MF, Chen GL. Estradiol-17β associated with the sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. J Exp Zool 1994; 268:53–58.
2. Chang CF, Yueh WS. Annual cycle of gonad histology and steroid profiles in the juvenile males and adult females of the protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture 1990; 91:179–196.
3. Lee MF, Huang JD, Chang CF. The development of ovarian tissue and female germ cells in the protandrous black porgy, *Acanthopagrus schlegeli*. Zool Stud 2008; 47:302–316.
4. Wu GC, DU JL, Lee YH, Lee MF, Chang CF. Current status of genetic and endocrine factors in the sex change of protandrous black porgy, *Acanthopagrus schlegeli* (Teleostean). Ann N Y Acad Sci 2005; 1040: 206–214.
5. Lee YH, Yueh WS, Du JL, Sun LT, Chang CF. Aromatase inhibitors block natural sex change and induce male function in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: possible mechanism of natural sex change. Biol Reprod 2002; 66:1749–1754.
6. Chang CF, Lau EL, Lin BY. Stimulation of spermatogenesis or of sex reversal according to the dose of exogenous estradiol-17β in juvenile males of protandrous black porgy, *Acanthopagrus schlegeli*. Gen Comp Endocrinol 1995; 100:355–367.
7. Chang CF, Lin BY. Estradiol-17β stimulates aromatase activity and reversible sex change in protandrous black porgy, *Acanthopagrus schlegeli*. J Exp Zool 1998; 280:165–173.
8. Wu GC, Tomy S, Chang CF. The expression of *nr0b1* and *nr5a4* during gonad development and sex change in protandrous black porgy fish, *Acanthopagrus schlegeli*. Biol Reprod 2008; 78:200–210.
9. Yao HH. The pathway to femaleness: current knowledge on embryonic development of the ovary. Mol Cell Endocrinol 2005; 230:87–93.
10. Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 2004; 131:933–942.
11. Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, Couty I, Batellier F, Cotinot C. Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev Dyn 2004; 231:859–870.
12. Baron D, Cocquet J, Xia X, Fellous M, Guiguen Y, Veitia RA. An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation. J Mol Endocrinol 2004; 33:705–715.
13. Nakamoto M, Matsuda M, Wang DS, Nagahama Y, Shibata N. Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun 2006; 344:353–361.
14. Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi K, Nagahama Y. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. Mol Endocrinol 2007; 21:712–725.
15. Soyal SM, Amleh A, Dean J. FIGa, a germ cell-specific transcription factor required for ovarian follicle formation. Development 2000; 127: 4645–4654.
16. Gen K, Okuzawa K, Kumakura N, Yamaguchi S, Kagawa H. Correlation between messenger RNA expression of cytochrome P450 aromatase and its enzyme activity during oocyte development in the red seabream (*Pagrus major*). Biol Reprod 2001; 65:1186–1194.
17. He CL, Du JL, Wu GC, Lee YH, Sun LT, Chang CF. Differential Dmrt1 transcripts in gonads of the protandrous black porgy, *Acanthopagrus schlegeli*. Cytogenet Genome Res 2003; 101:309–313.
18. England BG, Niswender GD, Mideley AR. Radioimmunoassay of estradiol-17β without chromatograph. J Clin Endocr Metab 1974; 38: 42–50.
19. Kime DE, Dolben IP. Hormonal changes during induced ovulation of the carp, *Cyprinus carpio*. Gen Comp Endocrinol 1985; 58:137–149.
20. Lau EL, Lin BY, Lee FY, Sun LT, Dufour S, Chang CF. Stimulation of testicular function by exogenous testosterone in male protandrous black porgy, *Acanthopagrus schlegeli*. J Fish Biol 1997; 51:327–333.
21. Coveney D, Shaw G, Renfree MB. Estrogen-induced gonadal sex reversal in the tammar wallaby. Biol Reprod 2001; 65:613–621.
22. Scheib D. Effects and role of estrogens in avian gonadal differentiation. Differentiation 1983; 23:S87–S92.
23. Merchant-Larios H, Ruiz-Ramirez S, Moreno-Mendoza N, Marmolejo-Valencia A. Correlation among thermosensitive period, estradiol response, and gonad differentiation in the sea turtle *Lepidochelys olivacea*. Gen Comp Endocrinol 1997; 107:373–385.
24. Kobayashi H, Iwamatsu T. Sex reversal in the medaka *Oryzias latipes* by brief exposure of early embryos to estradiol-17β. Zool Sci 2005; 22:1163–1167.
25. Kobayashi T, Kajiura-Kobayashi H, Nagahama Y. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. Cytogenet Genome Res 2003; 101:289–294.
26. Elbrecht A, Smith RG. Aromatase enzyme activity and sex determination in chickens. Science 1992; 255:467–470.
27. Hudson QJ, Smith CA, Sinclair AH. Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. Dev Dyn 2005; 233:1052–1055.
28. Belaid B, Richard-Mercier N, Pieau C, Dorizzi M. Sex reversal and aromatase in the European pond turtle: treatment with letrozole after the thermosensitive period for sex determination. J Exp Zool 2001; 290:490–497.
29. Wibbels T, Crews D. Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with temperature-dependent sex determination. J Endocrinol 1994; 141:295–299.
30. Guiguen Y, Baroiller JF, Ricordel MJ, Iseki K, Mcmeel OM, Martin SA, Fostier A. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol Reprod Dev 1999; 54:154–162.
31. Suzuki A, Tanaka M, Shibata N, Nagahama Y. Expression of aromatase

mRNA and effects of aromatase inhibitor during ovarian development in the medaka, *Oryzias latipes*. J Exp Zool Part A 2004; 301:266–273.
32. Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res Commun 1999; 264:230–234.
33. Amer MA, Miura T, Miura C, Yamauchi K. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). Biol Reprod 2001; 65:1057–1066.
34. Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci U S A 1998; 95:6965–6970.
35. Lee YH, Wu GC, Du JL, Chang CF. Estradiol-17β induced a reversible sex change in the fingerlings of protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: the possible roles of luteinizing hormone in sex change. Biol Reprod 2004; 71:1270–1278.
36. Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D. Foxl2 is required for commitment to ovary differentiation. Hum Mol Genet 2005; 14:2053–2062.