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The Expression of *amh* and *amhr2* Is Associated with the Development of Gonadal Tissue and Sex Change in the Protandrous Black Porgy, *Acanthopagrus schlegeli*[1]

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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, a bisexual gonad during the first 2 yr of life, and a male-to-female sex change (with vitellogenic oocytes) at 3 yr of age. In the present study, we investigated the possible roles of *amh* and *amhr2* in gonadal development in a nonmammalian model organism (protandrous black porgy), especially in relation to sex differentiation, testicular and ovarian growth, and sex change. Fish of various ages were treated with estradiol or an aromatase inhibitor to induce the fish to become female. Furthermore, a natural sex change (2^+-yr-old [>2 yr and <3 yr] fish) and a nonchemical method to surgically remove one of the pair of gonads to examine the possible roles of *amh* in the natural sex change were conducted. We present integrative in situ hybridization, immunohistochemical, cellular, and molecular data describing these phenomena. During gonadal sex differentiation, an increase in *amh* and *amhr2* expression was detected. Higher levels of *amh* and *amhr2* transcripts were observed in the testicular tissue when compared to the ovarian tissue in the bisexual gonad of 1^+-yr-old (>1 yr and <2 yr) fish. Transcripts of *amh* reached peak levels in November (prespermatogenesis period) and then declined to the lowest levels in January (spawning period). Chemical-induced ovarian tissue had very low *amh* transcript levels but high levels of *amhr2*. Active testes had significantly higher *amh* and *amhr2* expression levels as compared to inactive testes. In contrast, no difference in the expression of *amh* and *amhr2* between active and inactive ovarian tissues was found. Transcripts of *amh* were expressed in the somatic cells of the spermatogonia and vitellogenic oocytes, and *amhr2* was expressed in the somatic cells of the spermatogonia. Transcripts of *amh* decreased in the testicular tissue 5 mo before occurrence of the sex change into a female. In contrast, testicular *amh* expression remained high if the fish remained male. Human chorionic gonadotropin regulated *amh* and *amhr2* expression in the testicular tissue but not in the ovarian tissue. The present results suggest that *amh* plays important roles in early testicular and ovarian development, late ovarian growth (e.g., vitellogenic oocytes), and natural sex change in the protandrous black porgy.

amh, amhr2, *gonadal development, gonadal sex differentiation, oocyte development, sex change testis*

INTRODUCTION

Black porgy, *Acanthopagrus schlegeli* Bleeker, is a marine protandrous hermaphrodite fish that possesses a striking life cycle. These fish are functional males for the first 2 yr of life, but they transform into females during the third year of development [1, 2]. The differentiated bisexual gonad is observed in 4- to 5-mo-old fish [3]. Testicular tissues develop first and mature in the first reproductive cycle. The ovarian compartment remains dormant and contains few oogonia and primary oocytes in the first reproductive cycle until testicular tissue regresses during the postspawning season (March–May) [3]. The ovarian tissue grows and remains in the primary oocyte stage during the post- and nonspawning season (June–August) in 1^+-yr-old fish [3]. The ovarian tissue becomes the predominant tissue in the bisexual gonad until the prespawning season (September–December) in 1^+-yr-old fish (>1 yr and <2 yr), at which point it regresses concomitantly with the growth of testicular tissue. The testicular tissue resumes dominance and activity for the second reproductive season [3]. A similar pattern of ovarian development is observed during the post- and nonspawning season in 2^+-yr-old (>2 yr and <3 yr) fish, but the ovarian tissue does not regress in approximately 40–50% of the population. The fish will subsequently change sex by developing vitellogenic oocytes as the third reproductive season approaches [3, 4].

Increased plasma estradiol (E_2) levels are correlated with the natural sex change in the third reproductive cycle [1, 2], and the natural sex change is blocked by long-term administration of aromatase inhibitor (AI) [5]. Administration of E_2 for 1–2 mo resulted in regression of testicular tissue and decreased plasma 11-ketotestosterone [6]. A few vitellogenic oocytes were observed following treatment with E_2 for at least 5–6 mo in 1^+-yr-old black porgy [7]. Alternatively, after removal of testicular tissue from the bisexual gonad in 1^+-yr-old fish, the fish entered early sex change at the age of 2 yr [8–10]. These results suggest that the regression of testicular tissue is, indeed, a rate-limiting step in ovarian growth and natural sex change. The natural sex change proceeds in two steps: 1) testicular tissue begins to regress, and 2) the ovarian tissue becomes sensitive to endocrine signals, including estrogen [11] and gonadotropins [12], by expressing their target receptors. Therefore, the interplay between testicular and ovarian tissues is a key to understanding the natural sex change switch.

Anti-müllerian hormone (AMH), also known as müllerian-inhibitory substance, is the gonadal hormone that mediates regression of the müllerian ducts during male embryogenesis in mammals [13]. AMH is a member of the transforming growth factor (TGF-β) superfamily of glycoproteins, which includes inhibins and activins [14]. AMH signals through the type II AMH receptor (AMHR2) to regulate differentiation and the growth of target cells. AMHR2 is present on the cell membrane and is responsible for ligand binding [15]. Alternatively, three different type I AMH receptors (ALK2, also called ActRI;

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ALK3, also called BMPRI-A; and ALK6, also called BMPRI-B) are considered to mediate AMH response in target cells through AMHR2 binding [16].

In rodents, AMH transcripts are detectable in fetal testes during gonadal differentiation. This AMH signal is specific for the Sertoli cells of the testes during the embryonic period [17]. During male development, AMH expression is maintained at high levels, but it becomes low at the onset of germ cell meiosis in puberty [18]. In the female, the expression of AMH is first detected after birth and is restricted to granulosa cells [17, 18]. AMH is highly expressed in granulosa cells of primarily nonatretic preantral and small antral follicles [18]. In nonmammalian animals, *amh* also displays a conserved expression pattern. The expression levels of *amh* are consistently higher in males than in females during sex differentiation in chickens [19], alligators [20], turtles [21, 22], and fish (flounder [23], rainbow trout [24], zebrafish [25], and tilapia [26]). In contrast, in medaka, *amh* has no sexually dimorphic expression during gonadal differentiation but is highly expressed in the adult testis compared to the ovary [27]. These results suggest that *amh* signaling plays important roles in testicular development in vertebrates.

Alternatively, AMHR2 expression in Sertoli cells is concomitant with the initiation of spermatogenesis in immature testis [28]. AMHR2 is expressed abundantly in progenitors and immature Leydig cells as well as in Sertoli cells [29]. In females, AMHR2 is expressed in granulosa cells of nonatretic preantral and small antral follicles [28]. These results suggest that *amhr2* expression is dependent on a specific stage during gonadal development. In medaka, *amhr2* shows no sexually dimorphic expression during gonadal differentiation and, in adult fish, is expressed in Sertoli cells of the testis and granulosa cells of the ovary [27]. The profiles and possible roles of *amh* signaling and *amhr2* expression in relation to development of the gonad are not yet clear in hermaphrodite fish.

We proposed that *amh*/*amhr2* plays an important role in gonadal development, especially in relation to testicular differentiation and development as well as the late sex change in the protandrous black porgy. In the present study, protandrous black porgy exhibiting unique development of bisexual gonad and natural sex change were chosen to investigate *amh*/*amhr2* profiles in relation to the following: 1) gonadal sex differentiation (i.e., from undifferentiated to differentiated juveniles [0^+-yr-old; <1 yr fish]), given the functional mono-male sex pattern observed in this species during the first and second reproductive seasons; 2) E_2 activity during sex differentiation and gonadal development using exogenous E_2 and AI treatments in 0^+-yr-old; <1 yr fish; 3) the annual profile of gene expression in the bisexual gonad 0^+- to 3-yr-old (<3 yr) fish, especially related to natural sex change; 4) the testicular effects on the development of ovarian tissue in the bisexual gonad and the expression of *amh*/*amhr2* by in vivo surgical manipulation of testicular tissue and ovarian tissue; and 5) the effects of human chorionic gonadotropin (hCG) at different gonadal stages.

MATERIALS AND METHODS

Experimental Fish

Black porgy at different ages (0^+ to 3 yr) were used in the experiments. The experimental fish were acclimated to the pond environment at the university culture station in seawater and with a natural lighting system. Water temperatures ranged from 19 to 26°C. The fish were fed with commercial feed (Fwa Sou Feed Co.) 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.

Sample Collection

Before sampling, the fish were anesthetized in ethylene glycol monophenyl ether, and gonadal samples were removed for the examination of gene expression and histological studies. Gonadal tissues for gene expression studies were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. A piece of the gonadal tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 μm (hematoxylin-eosin and immunohistochemical staining) or 8 μm (in situ hybridization).

Experimental Design

Experiment 1: Gene profiles during male sex differentiation and E_2- and AI-induced female sex differentiation in undifferentiated fish. To clarify the influence of E_2 in testicular differentiation, we conducted E_2 and AI treatments in undifferentiated fish. Undifferentiated fish (age, 3 mo) were divided equally into three groups in June 2005: 1) control, 2) E_2-treated, and 3) AI-treated. Both E_2 (Sigma) and AI (1,4,6-androstatrien-3,17-dione; Steraloids, Inc.) were dissolved in ethanol and mixed with feed and water to obtain a final E_2 concentration of 6 mg/kg feed and a final AI concentration of 20 mg/kg feed. The treatments were given to the fish from June (age, 3 mo) to January of the following year (for 7 mo). The control group was given with feed devoid of E_2 or AI. Fish were collected regularly ($n = 8$–20 fish) in June (age, 3 mo; undifferentiated gonad), July (age, 4 mo; differentiating gonad), and August (age, 5 mo; differentiated gonad) for expression analyses ($n = 8$ fish/group), histology ($n = 6$ fish/group), and blood analysis ($n = 8$ fish/group). E_2 measurements were performed only using 6-mo-old fish.

Experiment 2: Effects of E_2 on morphological changes and gene profiles of the gonads of differentiated fish. To further clarify the effects of E_2 on gonadal development, differentiated fish (age, 5 mo) were equally divided into two groups in July: control and E_2-treated (6 mg/kg feed). E_2-treated fish were fed an E_2 diet from July to October, and E_2 administration was withdrawn and replaced by the control diet (devoid of E_2) from November to December. Fish samples ($n = 8$ fish/group) were collected monthly for genetic analysis and histology.

Experiment 3: Gene profiles in the gonads of differentiated fish. To examine the expression profile in the gonad at different reproductive stages, 1^+-yr-old black porgy were collected ($n = 8$) from May 2006 to January 2007. In addition, 1^+-yr-old black porgy were collected in August 2009 to examine the interplay of the bisexual gonad (active and inactive testis) and for genetic analysis, histology, TUNEL assay, and immunohistochemical staining with PCNA antiserum ($n = 6$ fish/group). Active (with strong Pcna and weak TUNEL staining) and inactive (with weak Pcna and strong TUNEL staining) gonadal tissues were collected for the experiment.

Experiment 4: Gene profiles during natural sex change. To examine the expression profile in the gonad during natural sex change, 2^+-yr-old black porgy were collected regularly ($n = 8$ fish) at 2-mo intervals from May 2002 (after the second spawning) to January 2003 (after the sex change). Testicular tissue was collected to examine the sex of the fish and for genetic analysis.

To further identify the fate of the bisexual gonad (continued of male function or natural sex change) in relation to the expression of *amh*/*amhr2*, 2^+-yr-old fish were marked with chips (June 2007, nonspawning season), and a surgical technique was used to collect the right side of the gonad in July and August (nonspawning season) for genetic analysis. Next, the fish were killed, and their sex was checked in January 2008.

Experiment 5: Short-term effects of hCG on the genetic profile in gonadal tissue. To examine the short-term effects of hCG on the ovarian genetic profile, 1^+-yr-old black porgy were divided equally into three groups ($n = 5$–10 fish/group) in September 2009: 1) fish with inactive testicular tissue and active ovarian tissue, 2) fish with active testicular tissue and inactive ovarian tissue, and 3) fish with active ovarian tissue without testis. The testicular tissue in the group ''active ovarian tissue without testis'' was removed from the bisexual gonad in August 2009 by a surgical approach as described previously [9]. The hCG (2 IU/g body wt) was injected (i.p.) on Days 0, 2, and 4, and fish gonads were collected on Day 5 for genetic and histological analyses. No histological changes in the gonad were observed after short-term hCG treatment.

Immunohistochemical Staining

According to a previous study [8], antiserum against human PCNA (sc-7907; Santa Cruz Biotechnology, Inc.) detects a specific and single protein band corresponding to black porgy Pcna. For immunochemical staining, the PCNA antibody was developed overnight. This was followed by incubation

with biotinylated anti-rabbit immunoglobulin G (H+L; Vector Laboratories, Inc.). Color formation was amplified with an ABC kit (avidin-biotin-peroxidase; Vector Laboratories, Inc.) and DAB (3,3′-diaminobenzidine; Sigma). Finally, the tissue section was counterstained with hematoxylin.

TUNEL Staining

The paraffin section was rehydrated in a PBS buffer and incubated in an equilibration buffer (Promega) at room temperature. DNase I-treated slides were used as a positive control. While the sections were equilibrating, they also were incubated in recombinant terminal deoxynucleotidyl transferase enzyme and biotinylated nucleotide at 37°C for 1 h, followed by termination in 1× SSC (0.15 M sodium chloride and 0.015 M sodium citrate) for 15 min at room temperature (Promega). Slides were then rinsed three times in PBS and covered with 3% H_2O_2. Color formation was amplified with Streptavidin HRP and DAB (Promega).

In Situ Hybridization

In situ hybridization of tissue sections was performed as described previously [8] using digoxigenin-labeled probes for *amh* and *amhr2*. The probe was developed overnight, as a standard in our laboratory. Immunostaining was performed with preabsorbed alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche) at room temperature. Finally, the NBT/BCIP (4-nitroblue tetrazodiym chloride/5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt) Detection System (Sigma) was used to detect *amh* and *amhr2* expression.

Reverse Transcription-Polymerase Chain Reaction

Total RNA extracted from the gonad of the representative fish was reverse transcribed to the first-strand cDNA using Superscript III (Invitrogen) with the oligo$(dT)_{12–18}$ primers (Promega) according to the manufacturer's protocol. The first-strand cDNA was ready for the PCR and real-time PCR. Specific primers were designed for the PCR for *amh* (sense: 5′-GCAGACTATGATG CAGATAA-3′; antisense: 5′-ACCAGTGGGACAGGACATGTG-3′), *amhr2* (sense: 5′-GATCTGAGCAGCTTCAACGTGCT-3′; antisense: 5′-GTCCCAACAGTCCGTCAGGATCT-3′), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; sense: 5′-GCATCTTGCACGGCTAACT-3′; antisense: 5′-CGGCGCCGGCATCGAAGAT-3′); *gapdh* was used as an internal control. PCR conditions were as follows: 94°C for 30 sec, 54°C for 1 min, and 72°C for 1.5 min. The number of PCR cycles underwent preliminary testing and was in the range of the linear curve for the relationship between the number of cycles and the amount of PCR product.

Quantification of Gene Transcripts by Quantitative Real-Time PCR Analysis

The first-strand cDNA was used for the quantitative real-time PCR analysis. Quantitative real-time PCR analysis of gene transcripts of *amh* (GU256046), *amhr2* (GU256045), *cyp19a1a* (P450arom, AY870246), *cyp11b2* (P45011β, EF423618), *star* (AY870248), *cyp11a1* (AY870246), and *gapdh* (DQ399798) were conducted according to the methods described in previous studies [9, 11]. Specific primers for *amh* (sense: 5′-GCCTCACTGTGTCCCTTGAAA-3′; antisense: 5′-ACCAGTGGGACAGGACATGTG-3′), *amhr2* (sense: 5′-CTG TGCCCTGTGTGATTTCG-3′; antisense: 5′-TGCTGTGTCGTTCTG CCTGTA-3′), and other genes [9] were designed for the quantitative real-time PCR. Quantifications of standards, samples, and controls were conducted simultaneously by real-time PCR (GeneAmp 7500 Sequence Detection System; Applied Biosystems) with SYBR green I as a dsDNA minor-groove binding dye. The standard curves of log (transcript concentrations) versus 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 the internal control *gapdh* and then normalized.

Data Analysis

All data are expressed as the 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 significant differences ($P < 0.05$) between two treatments.

RESULTS

Gene Profiles During Male Sex Differentiation and E_2- and AI-Induced Female Sex Differentiation

The differentiated gonad was observed in 4- to 5-mo-old fish. E_2 and AI were given to the fish before gonadal differentiation (Fig. 1A). Ultimately, after 3 mo of E_2 administration (6-mo-old fish) and 4 mo of AI administration (7-mo-old fish), the fish had well-developed ovarian tissue and regressed testicular tissue (Fig. 1B). After 3 mo of treatment, plasma E_2 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, E_2 group, and AI group, respectively ($P < 0.05$).

In juvenile fish with differentiating and differentiated testis (age, 4 and 5 mo, respectively), *amh* expression significantly increased (1.7- and 2.7-fold, respectively) compared to the undifferentiated gonad (3 mo) and reached to a peak ($P < 0.05$) in the gonad of 5-mo-old fish (Fig. 1C). Decreased levels of *amh* transcripts were found in the E_2- and AI-treated gonadal tissue compared with the gonads in the control group (Fig. 1C). E_2 caused lower *amh* transcript expression than AI in this experiment (446-fold decrease in the E_2 group and 4-fold decrease in the AI group in 5-mo-old fish, $P < 0.05$) (Fig. 1C); however, *amh* did not change among undifferentiated gonads (3.5 mo), differentiating gonads (4 mo), and differentiated ovarian tissue (5 mo) in the E_2- and AI-treated groups (Fig. 1C).

Transcripts of *amhr2* significantly increased ($P < 0.05$) in differentiating (4 mo) and differentiated (5 mo) gonads compared to the undifferentiated gonad in 3- or 3.5-mo-old fish in the control (increase of 2.9- and 4.1-fold, respectively), E_2-treated (increase of 3.4- and 7-fold, respectively), and AI-treated (increase of 1.3- and 7-fold, respectively) groups (Fig. 1C). E_2 caused higher ($P < 0.05$) *amh2* transcript levels than in the control and AI-treated group in 5-mo-old fish (Fig. 1C).

Effects of E_2 on Morphological Changes and Gene Profiles of the Gonads of Differentiated Juvenile Fish

After 1 mo of E_2 administration, the development of ovarian tissue was detected in fish in August. After 3 mo of E_2 administration, the fish had well-developed ovarian tissue and regressed testicular tissue. E_2 administration was withdrawn (E_2 termination) after October. These fish that had an E_2-induced early sex change had a reversible sex change after E_2 termination. After E_2 termination, testicular tissue regenerated in concert with the regression of ovarian tissue, and the fish became males during the first reproductive period.

Quantitative real-time PCR data showed that *amh* transcripts declined in ovarian tissue after 1 mo of E_2 administration (27.6-fold decrease, $P < 0.05$) and remained low ($P < 0.05$) compared to the testicular tissue in the controls (Fig. 2). The expression levels of *amh* did not change in the ovarian tissue of E_2-fed (August–October) and E_2-terminated (November–December) periods. Levels of *amhr2* transcripts in the ovary of E_2-fed group in September were lower (2.3-fold decrease, $P < 0.05$) than the levels in the testicular tissue of the control (Fig. 2). The expression levels of *amhr2* did not change ($P < 0.05$) in the ovarian tissue of the E_2-fed (August–October) and E_2-terminated (November–December) periods (Fig. 2). In November, the relative expression among the testicular tissue in the control, regenerated testicular tissue in E_2-terminated group, and ovarian tissue in E_2-terminated group for *amh* was 1.00 ± 0.20, 1.13 ± 0.23, and 0.06 ± 0.01, respectively; for *amhr2*, the relative expression was 1.00 ± 0.18, 1.67 ± 0.18, and 1.95 ± 0.19, respectively.

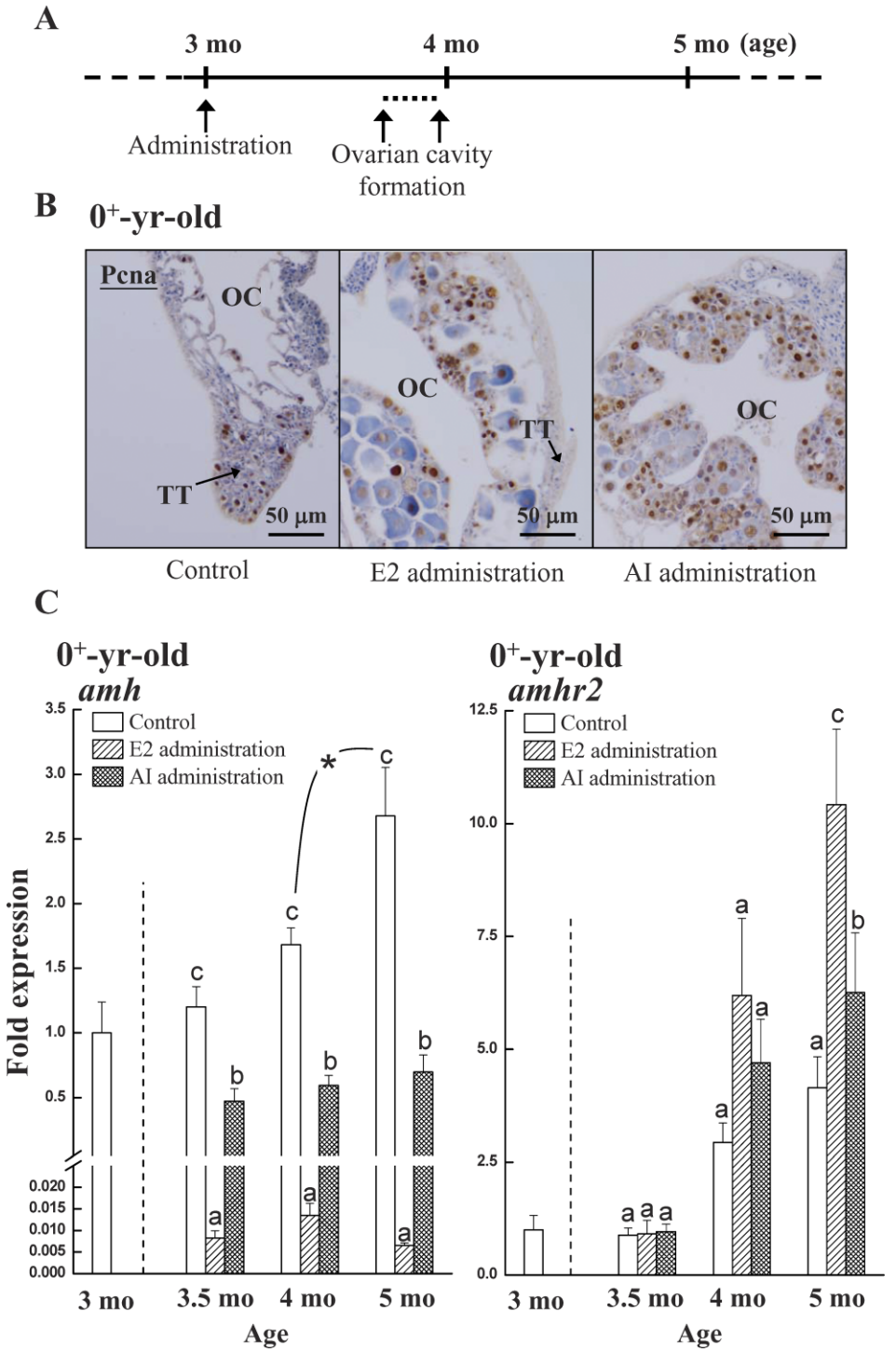


FIG. 1. Gonadal development with cell proliferation and gene expression in E_2- and AI-treated 0^+-yr-old undifferentiated fish. **A**) A time course of ovarian development through manipulation of E_2 and AI administration. E_2 or AI was given to fish from 3 to 11 mo of age. **B**) Transverse sections of gonadal tissue immunohistochemically stained for Pcna (brown) and counterstained with hematoxylin (purple) in the gonad of the control fish (primarily testicular tissue), E_2-treated fish (primarily ovarian tissue in 6-mo-old fish), and AI-treated fish (primarily ovarian tissue in 7-mo-old fish). **C**) The mRNA levels of *amh* and *amhr2* as measured by real-time PCR in the gonadal tissue in 3- and 3.5-mo-old (undifferentiated), 4-mo-old (differentiating), and 5-mo-old (differentiated) fish. The values were calibrated with the internal control, *gapdh*. The control values of *amh* and *amhr2* in 3-mo-old fish (before E_2/AI administration) were defined as one. Each value is expressed as the mean ± SEM (n = 8 for each value). Different letters represent statistical difference ($P < 0.05$) in the same group. Asterisk indicates significant difference ($P < 0.05$) between the groups in 4-mo-old and 5-mo-old fish. OC, ovarian tissue; TT, testicular tissue. Bar = 50 μm.

Gene Profiles in the Gonad of 1^+-Year-Old Differentiated Fish

After the first spawning season, a bisexual gonad with regressed testicular tissue and developed ovarian tissue (regressed testicular tissue with yellow bodies; ovarian tissue became the dominant tissue with the primary oocytes and ovarian lamellae) was found in 1^+-yr-old fish older during March to August (male to female) (Fig. 3A). Later, the ovarian tissue regressed concomitantly with the development of testicular tissue, and the number of spermatogonia increased from August to November (female to male) (Fig. 3A). Spermatogenesis (appearance of spermatogonia, spermatocytes, spermatids, and/or sperms) and maturation appeared in the testicular tissue in November and January (the second spawning season), respectively (Fig. 3A).

Quantitative real-time PCR data showed that *amh* transcripts significantly increased in August (3.4-fold increase) and peaked in November (7-fold increase) as compared to the value in testicular tissue in May (Fig. 3B). Later, *amh* expression dramatically decreased in January in testicular tissue (Fig. 3B). Testicular tissue had significantly higher levels of *amh* (13- to 40-fold, $P < 0.05$) and *amhr2* (145- to 412-fold, $P < 0.05$) transcripts than in ovarian tissue (Fig. 3B). Levels of *amh* expression did not change in the ovarian tissue of 1^+-yr-old fish

(Fig. 3B). The expression of *amhr2* in testicular tissue remained at high levels from May to November but decreased (2.5-fold) in January during the second spawning period (Fig. 3B). In ovarian tissue, *amhr2* expression was higher in May and then decreased in the period from August to January (Fig. 3B).

Comparison of amh *and* amhr2 *Expression in Gonadal Tissue Between Inactive and Active Stages of 1$^+$-Year-Old Fish*

Development of a bisexual gonad is a dynamic process in black porgy. The active and inactive gonadal tissues were classified according to the histological data related to Pcna and TUNEL staining. According to immunohistochemical Pcna staining and TUNEL staining, much less Pcna staining was observed in the inactive testis (Fig. 4Aa) as compared to the strong Pcna staining in many oogonia (Fig. 4Ab) and weak TUNEL staining (Fig. 4Ac) in the active ovary in the bisexual gonad in 1$^+$-yr-old fish. In contrast, the active testis (Fig. 4Ad) had stronger signals of Pcna staining in spermatogonia as compared to fewer signals of Pcna staining in the inactive ovary (Fig. 4Ae), whereas strong TUNEL staining for apoptosis was observed in the inactive ovary (Fig. 4Af) of the bisexual gonad in 1$^+$-yr-old fish.

The expression of *amh* and *amhr2* significantly increased (4.6- and 3-fold, respectively; both $P < 0.05$) in active testicular tissue compared to inactive testicular tissue (Fig. 4B). The expression levels of *amh* and *amhr2* were not different between active and inactive ovarian tissues (Fig. 4B).

Expression Profiles of amh *and* amhr2 *in 0$^+$- to 3-Year-Old Fish*

The annual profiles of the development of testicular and ovarian tissues in the bisexual gonad from fish at the undifferentiated stage to 3 yr of age in comparison with the profiles of *amh* and *amhr2* expression are shown in Figure 5. The levels of testicular *amh* transcripts were high in differentiated 0$^+$- and 1$^+$-yr-old fish, then decreased in stages of spermatogenesis and maturation in 1-, 2-, and 3-yr-old male fish (Figs. 1–3 and 5B). Conversely, ovarian *amh* expression was low in 0$^+$-, 1$^+$-, and 2$^+$-yr-old fish (before sex change) and significantly increased in 3-yr-old fish in the ovarian tissue with vitellogenic oocytes (sex change) (Figs. 3 and 5B). Expression of *amh* decreased in the mature oocytes during the third spawning season (Fig. 5B). The levels of *amhr2* transcripts were consistently high in the testicular tissue of 1$^+$- and 2$^+$-yr-old fish (Figs. 3 and 5B). In contrast, *amhr2* transcript levels were consistently low in the ovarian tissue of 1$^+$- and 2$^+$- to 3-yr-old fish (Figs. 3 and 5B).

Localization of amh *and* amhr2 *Expression*

In situ hybridization for *amh* (antisense probe in Fig. 5, Ca–Cd; sense probe in Fig. 5Ce) and *amhr2* (antisense probe in Fig. 5, Cf–Ci; sense probe in Fig. 5Cj) are shown in Figure 5C. In situ hybridization demonstrated that *amh* (Fig. 5, Ca–Cc) and *amhr2* transcripts (Fig. 5, Cf–Ch) were expressed in the surrounding cells (Sertoli cells) of spermatogonia in early testis (*amh* in Fig. 5Ca; *amhr2* in Fig. 5Cf), in developed testis (*amh* in Fig. 5Cb; *amhr2* in Fig. 5Cg), and in the Leydig cells of the maturing testis (*amh* in Fig. 5Cc, *amhr2* in Fig. 5Ch). Ovarian *amh* (Fig. 5Cf), but not *amhr2* (Fig. 5Ci), transcripts significantly appeared in the follicle cells of vitellogenic oocytes.

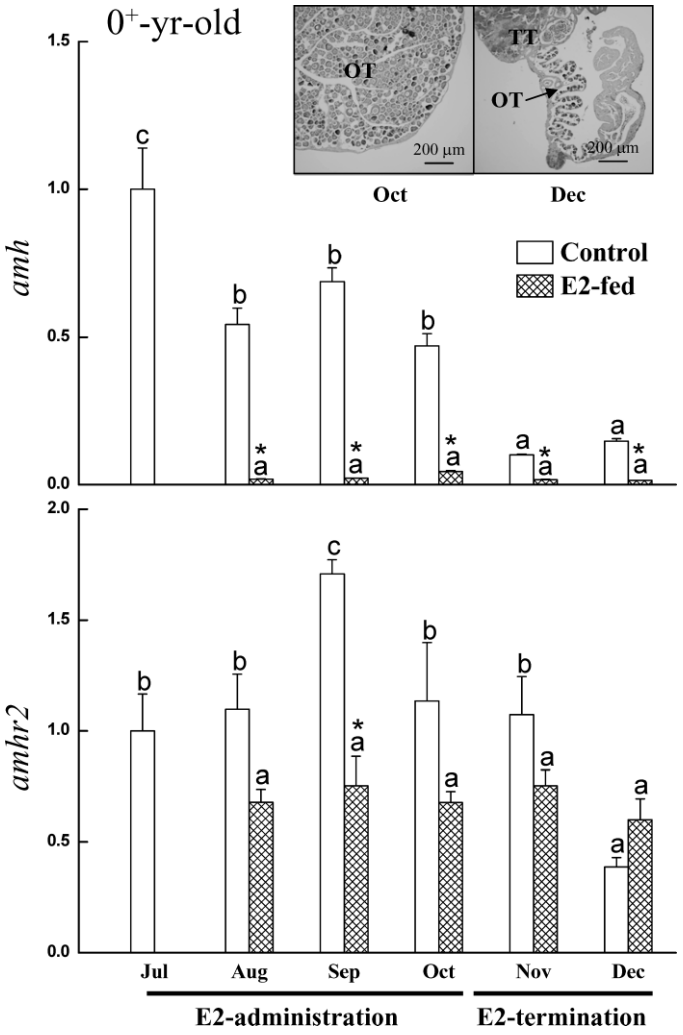


FIG. 2. Relative expression profiles of *amh* and *amhr2* during ovarian development (E_2 administration during July–October; ovarian tissue in October) and regression (E_2 termination during November–December; a bisexual gonad with testicular and ovarian tissues in December) in 0$^+$-yr-old differentiated fish. Expression of *amh* and *amhr2* in the gonad was measured by real-time PCR from July to December. The values were calibrated with the internal control, *gapdh*. The first value in the control testis in July was defined as one. Each value is expressed as the mean ± SEM (n = 8 for each value). Different letters represent statistical difference ($P < 0.05$) in the same group. An asterisk indicates significant difference ($P < 0.05$) between the groups in 3.5-mo-old and 5-mo-old fish. OT, ovarian tissue; TT, testicular tissue. Bar = 200 μm.

Testicular amh *Expression Profiles in 2$^+$- to 3-Year-Old Fish During Natural Sex Change*

During natural sex change, quantitative real-time PCR data showed that the variation of testicular *amh* transcripts was small in 1$^+$-yr-old fish (Fig. 3B) and 2$^+$-yr-old fish (Fig. 6A) in May, but the variation became large in 2$^+$-yr-old fish in July, September, and November (Fig. 6A). Levels of *amh* transcript significantly decreased during the spawning season (January) in 3-yr-old males (Fig. 6A).

To further investigate the possible roles of *amh* in natural sex change, one side of the gonads was surgically removed in July and August from 2$^+$-yr-old fish, and the fish were individually identified by chip label to trace the fish stage (either natural sex change to female or continued male function) during the third spawning season (5 mo after the

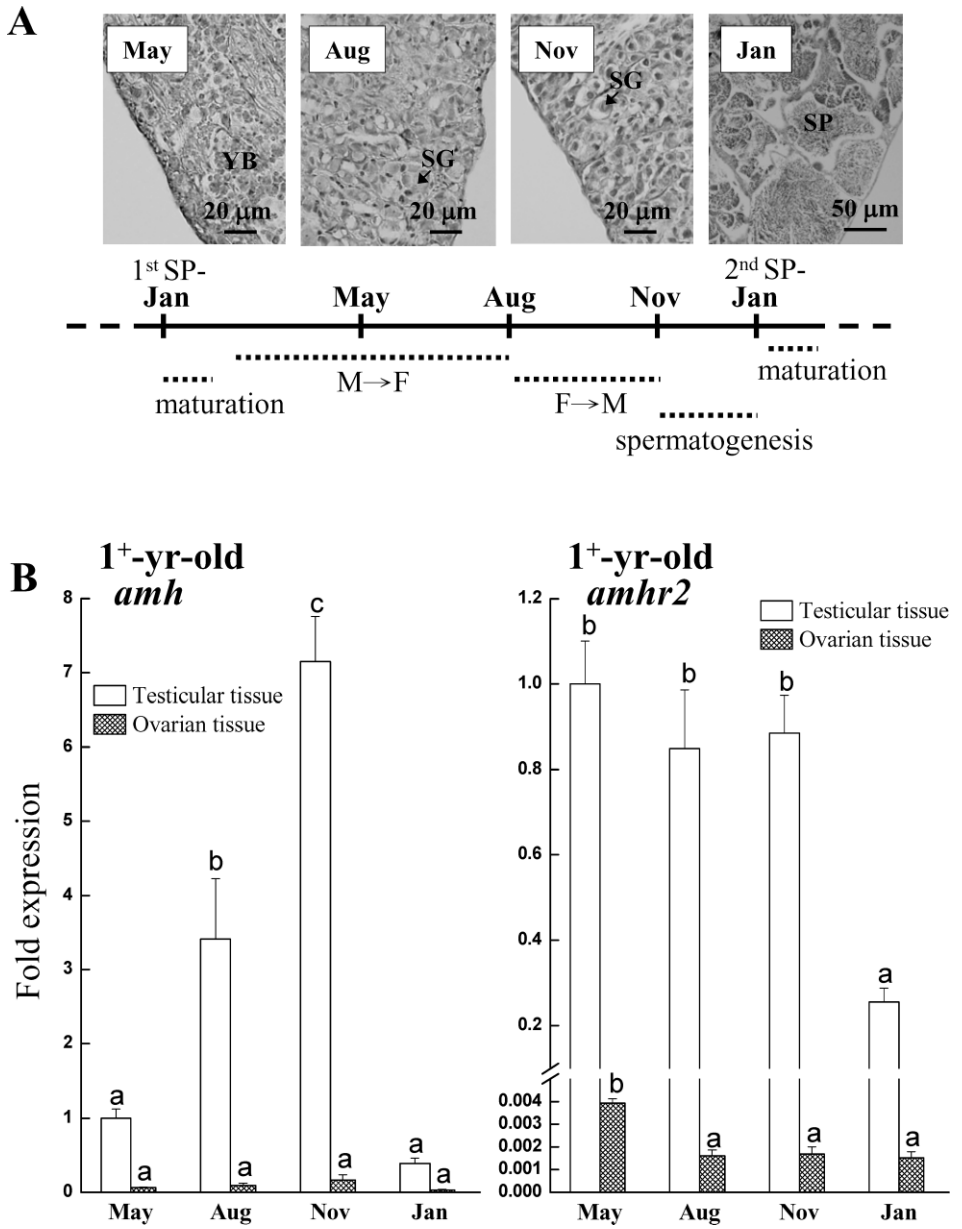


FIG. 3. Relative expression profiles of *amh* and *amhr2* at different reproductive stages in 1^+-yr-old fish. **A**) A time course of bisexual gonadal development in 1^+-yr-old fish (male phase to female phase [M → F]: the fish with the yellow bodies in the regressed testicular tissue, dominant ovarian tissue and primary oocytes; female phase to male phase [F → M]: the fish with the regression of ovarian tissue, growth of testicular tissue with spermatocytes and sperms). **B**) Expression of *amh* and *amhr2* in the gonad measured by real-time PCR from May to January of the next year. The values were calibrated with the internal control, *gapdh*. The first value in control testis in May was defined as one. Each value is expressed as the mean ± SEM (n = 8 for each value). Different letters represent statistical difference ($P < 0.05$) in the same group. 1st SP, the first spawning season; 2nd, the second spawning season; SG, spermatocyte; SP, sperm; YB, yellow body. Bar = 20 μm (May, Aug, and Nov) and 50 μm (Jan).

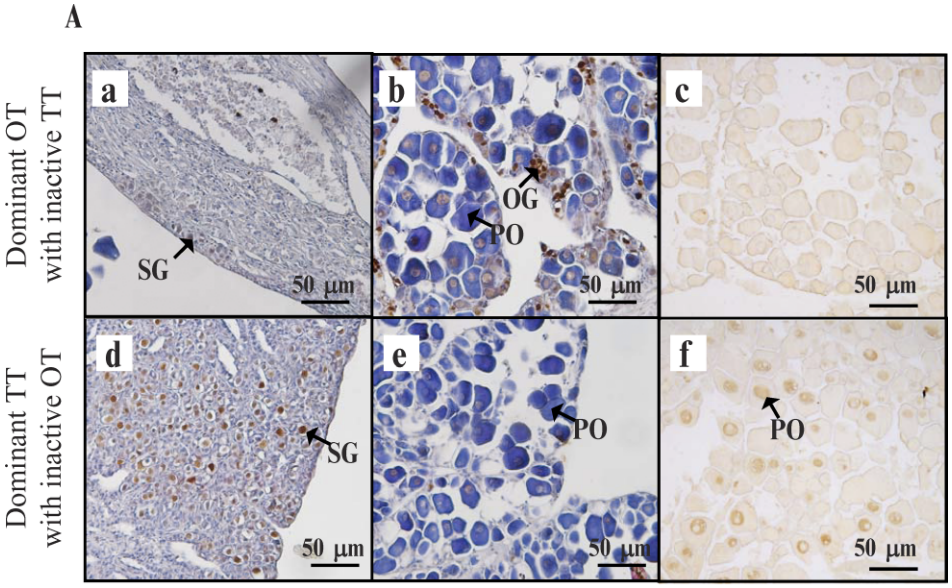
surgical operation). Quantitative real-time PCR data demonstrated that testicular *amh* transcript levels were significantly higher (1.7-fold, $P < 0.05$) in fish that maintained male function compared to the fish in which a natural sex change occurred (Fig. 6B). In contrast, testicular *amhr2* and *dmrt1* and ovarian *amh* and *amhr2* were not significantly different between the two groups (continued a male vs. sex change to a female) in the testis of 2^+-yr-old fish in the period from July to August (data not shown).

Effects of hCG on Gene Expression in the Gonad

The values of each hCG-treated group (inactive testicular tissue, active testicular tissue, inactive ovarian tissue, active ovarian tissue, and active ovarian tissue without testis) were calibrated by the respective value of the control fish. The value of the control fish was defined as one (the dotted line in the Fig. 7). The expression of *amh* transcripts was stimulated in the inactive testicular tissue but suppressed in the active testicular tissue after hCG treatment as compared to the respective control (Fig. 7A). Treatment with hCG caused the downregulation of *amhr2* in the inactive and active testicular tissues (Fig. 7A). The expression of *star* increased in two groups (increases of 5.4- and 7.8-fold, $P < 0.05$) as compared to the respective control. Conversely, *cyp11a1* transcripts significantly increased in active testis (4.7-fold) but not in inactive testis after hCG treatment (Fig. 7A). Ovarian *amh* and *amhr2* were not changed in different groups after hCG treatment as compared to the respective control (Fig. 7B). The expression level of *star* significantly increased in the active ovary (with or without testes; increase of 3.6- and 3.4-fold, respectively; $P < 0.05$) but not in the inactive ovary after hCG treatment as compared to the control (Fig. 7B). Conversely, *cyp11a1* transcripts significantly increased in the inactive ovary (152-fold, $P < 0.05$) but not in the active ovary (with or without testes) after hCG treatment as compared to the control (Fig. 7B).

DISCUSSION

Black porgy undergo gonadal (testicular and ovarian) sex differentiation in the juvenile fish, mono-male growth and



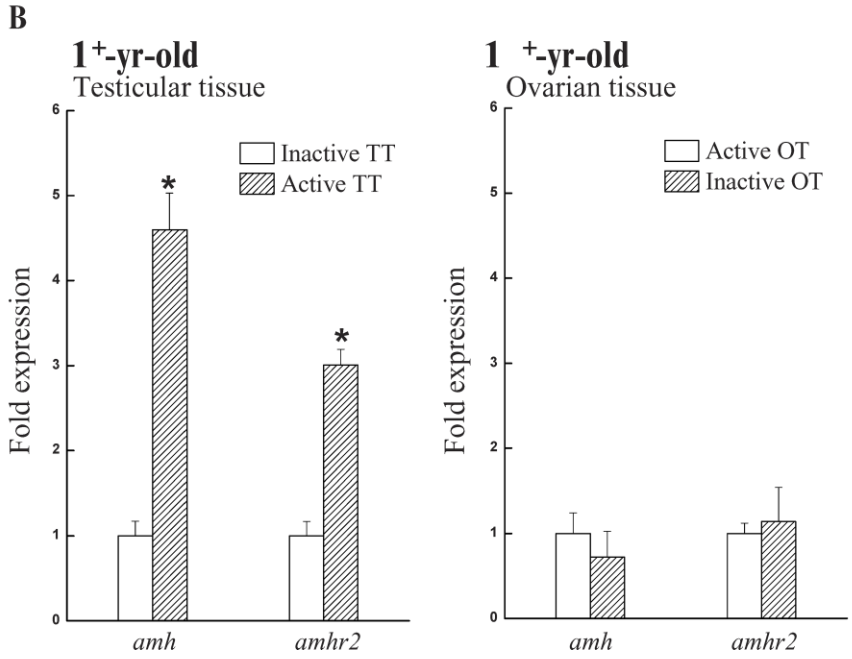


FIG. 4. Cellular stages and relative gene profiles of *amh* and *amhr2* in the gonad of 1^+-yr-old fish. **A**) Transverse sections of gonadal tissue stained for cellular proliferation (**a**, **b**, **d**, and **e**) and apoptosis (**c** and **f**) in the gonad. For cellular proliferation, the sections were immunohistochemically stained for Pcna (brown) and counterstained with hematoxylin (purple). For apoptosis, a TUNEL assay was conducted to identify degraded DNA (red). **B**) Expression of genes at the different stages of the gonad measured by real-time PCR. The values were calibrated with the internal control, *gapdh*. The first value in the inactive testis and in the active ovary was defined as one. Each value is expressed as the mean ± SEM (n = 5–8 for each value). An asterisk indicates a statistical difference ($P < 0.05$) between active and inactive tissues. OG, oogonium; OT, ovarian tissue; PO, primary oocyte; SG, spermatogonium; TT, testicular tissue. Bar = 50 μm.

maturation with a bisexual gonad, and a subsequent natural sex change to the female sex; therefore, they are a unique model to study the mechanism of interplay in testicular and ovarian development. In this report, we examined the consequences of *amh* in testicular differentiation and development and the potential interplay between testicular and ovarian tissue in black porgy.

The gene *amh* has long been considered important for male sex differentiation in vertebrates. Based on quantitative real-time PCR data, transcription levels of *amh* and *amhr2* significantly increase after testicular differentiation. These data showed that *amh* and *amhr2* correlate with testicular differentiation and development in black porgy. After gonadal differentiation, *amh* and *amhr2* continued to be highly expressed in testicular tissue of 1^+- and 2^+-yr-old fish. The expression of gonadal *amh* is higher in males than in females during sex differentiation (e.g., in mammals [18, 28], chickens [19], reptiles [20, 22], and fish [23, 25, 26]). However, AMH-deficient male mice have testes that are fully descended and that produce functional sperm [30]. Conversely, in medaka fish, *amh* and *amhr2* have no sexually dimorphic expression during gonadal differentiation, and the loss of function of *amh* and *amhr2* results in the suppression of germ cell proliferation during gonadal differentiation in both sexes [31]. In medaka fish with hotei mutation (*amhr2*-deficient), more than 50% of fish undergo sex reversal [32]. These data indicate that the normal function of *amh* and *amhr2* is important to maintain germ cell proliferation by mitosis in the male gonad.

Furthermore, to investigate in more detail the possible functions of *amh* and *amhr2*, we examined the expression patterns of *amh* and *amhr2* in differentiated black porgy. The transcripts of *amh* and *amhr2* were highly expressed in testicular tissue compared to ovarian tissue in 1^+- and 2^+-yr-old black porgy. In addition, testicular *amh* and *amhr2* transcripts appeared in spermatogonia-surrounding cells, and the expression of *amh* and *amhr2* transcripts significantly increased during germ cell mitosis (active testis) and peaked before germ cell meiosis (spermatogenesis in November). The

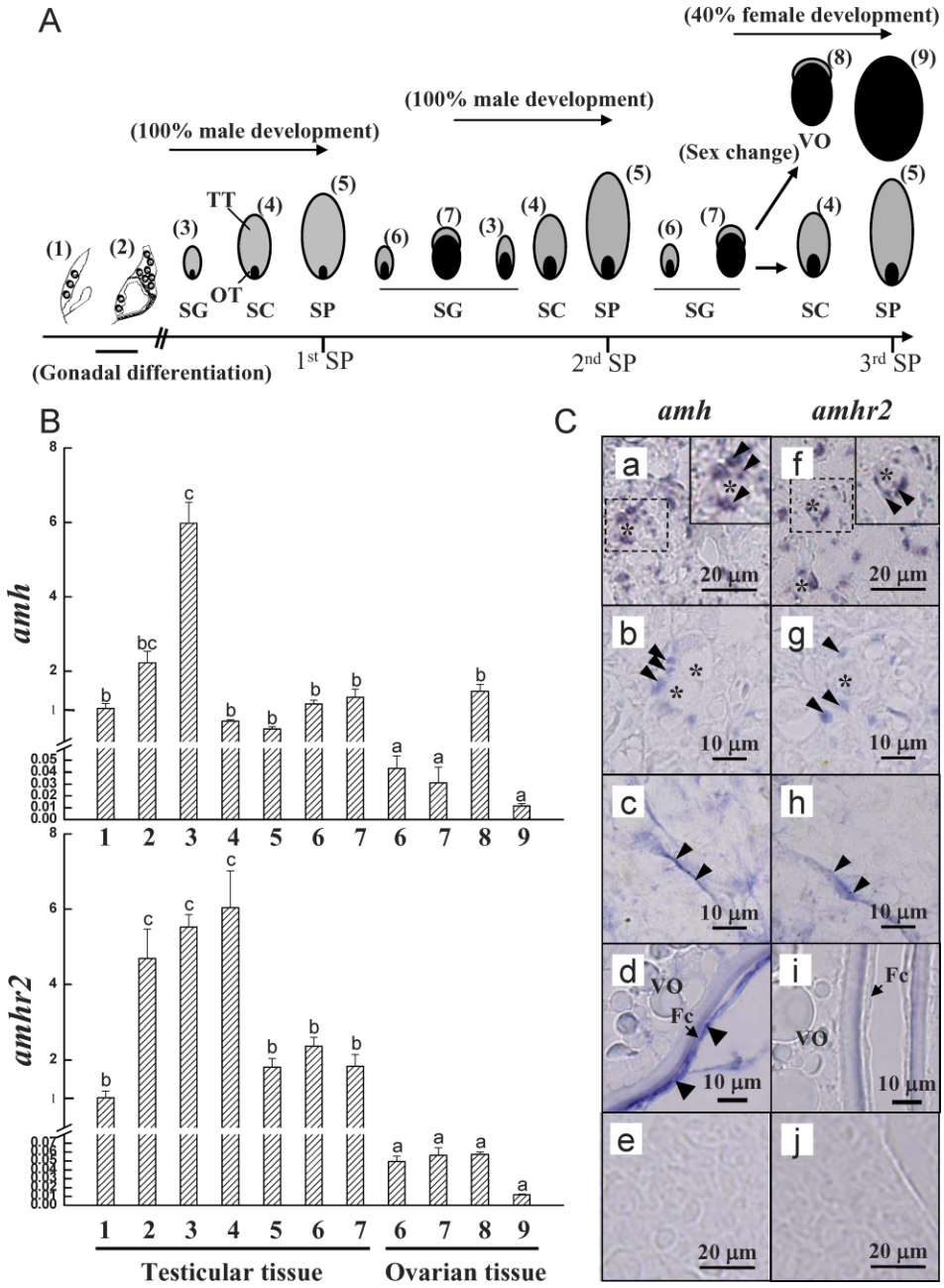


FIG. 5. Gene profiles (*amh* and *amhr2*) in the testicular and ovarian tissues during natural sex change from 0^+- to 3-yr-old fish. **A**) Annual profiles of gonadal tissue from 0^+- to 3-yr-old fish in relation to the development of bisexual gonad (testicular tissue [TT] in gray; ovarian tissue [OT] in dark). The gonadal stages were defined as follows: 1, undifferentiated gonad; 2, differentiating gonad; 3, active testis with spermatogonia and inactive ovary with the primary oocytes (0^+- and 1^+-yr-old fish); 4, active testis with spermatogonia and inactive ovary with the primary oocytes (0^+-, 1^+-, and 2^+-yr-old fish); 5, mature testis (1-, 2-, and 3-yr-old fish); 6, regressed testis and active ovary with the primary oocytes (1^+- and 2^+-yr-old fish); 7, inactive testis and active ovary with the primary oocytes (1^+- and 2^+-yr-old fish); 8, degenerated testis and active ovary with vitellogenic oocytes (2^+-yr-old fish); and 9, mature ovary (3-yr-old fish). **B**) Relative *amh* and *amhr2* transcripts at different gonadal stages in 0^+- to 3-yr-old fish. The values were calibrated with the internal control, *gapdh*. The first value in the undifferentiated fish (stage 1) was defined as one. The stages of the gonad (stages 1–9) are as described in **A**. **C**) In situ hybridization of *amh* and *amhr2* with antisense (**a**–**d** and **f**–**i**) and sense (**e** and **j**) probes at different gonadal stages: **a** and **f**) Sertoli cells of spermatogonia of differentiating testis in 0^+-yr-old fish; **b** and **g**) Sertoli cells of spermatogonia in the developed testicular tissue in 0^+-yr-old fish; **c** and **h**) Leydig cells in the maturing testis in 1^+-yr-old fish; **d** and **i**) follicle cells in the natural sex change fish with vitellogenic oocytes of 3-yr-old fish; and **e** and **j**) sense probe with *amh* and *amhr2* in the maturing testis of 1^+-yr-old fish. Different letters represent statistical difference ($P < 0.05$) in the same group. Fc, follicle cell; SC, spermatocyte; SG, spermatogonium; SP, sperm; VO, vitellogenic oocyte; *, spermatogonium; 1st SP, first spawning season; 2nd SP, second spawning season; 3rd SP, third spawning season. Positive staining is indicated by a triangle in black. Bar = 20 μm (**C**: **a**, **e**, **f**, and **j**) and 10 μm (**C**: **b**–**d** and **g**–**i**).

level of *amh* transcripts declined in mature testes during the spawning season. In other vertebrates (e.g., in mammals [18, 28, 33] and fish [23, 34]), transcription levels of *amh* were comparatively high in immature testes, decreased when spermatogenesis was initiated, and were the lowest during spermatogenesis. In the rat, these *Amh*-deficient testes also had Leydig cell hyperplasia, and the immature Leydig cells overproliferated [30]. These data indicate that *amh* and *amhr2*

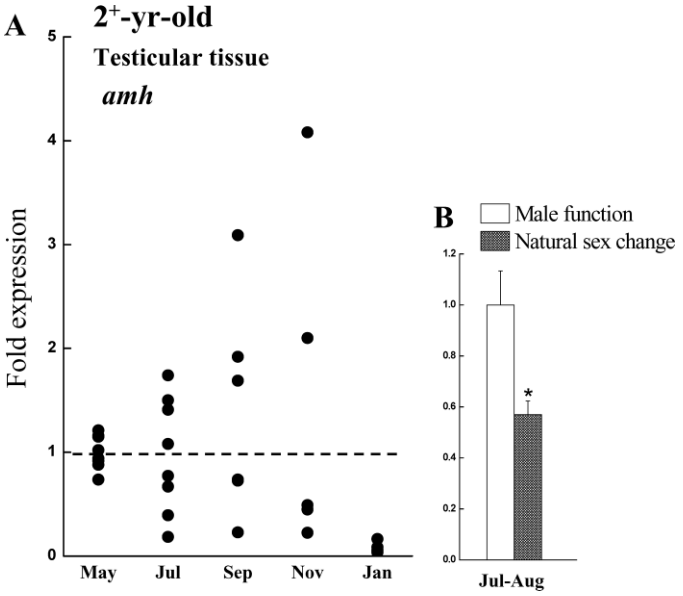


FIG. 6. Relative expression profiles of *amh* during natural sex change in 2^+- to 3-yr-old fish. **A**) Expression of *amh* in the gonad measured by real-time PCR from May to January of the next year. Each dark circle represents a single fish value. The value in testicular tissue in May was defined as one. **B**) The gonad in the right side was sampled by surgical operation in July and August, and sexual status was identified in January of the next year. Expression of *amh* in the gonads (collected from July and August) was measured by real-time PCR. The value in testicular tissue during the nonspawning season (July and August) that maintained male function during the third spawning season (January) was defined as one. Each value was expressed as the mean ± SEM. An asterisk indicates a statistical difference ($P < 0.05$) between the two groups (remained male function vs. natural sex change) (n = 5–8 for each value).

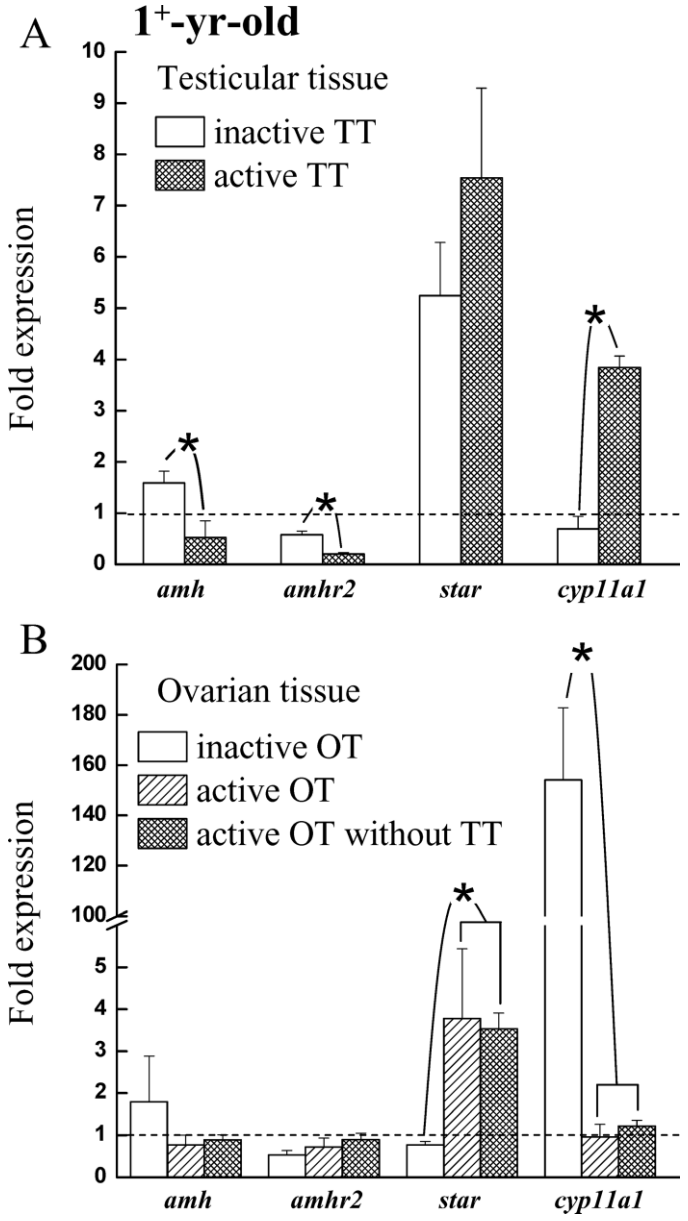


FIG. 7. Relative responses of gene transcripts at different stages of gonad of 1^+-yr-old fish after hCG injection. The hCG (2 IU/g body wt) was injected (i.p.) on Day 0, 2, and 4, and fish gonads were collected on Day 5 for genetic analyses. The values measured by real-time PCR were calibrated with the internal control, *gapdh*. Relative responses of hCG-injected fish were calibrated with respect to the control fish. The control value in each group was defined as one and represented as a dotted line. Each value was expressed as the mean ± SEM. An asterisk indicates a significant difference ($P < 0.05$) in the same group (n = 5–8 for each value). OT, ovarian tissue; TT, testicular tissue.

are important not only to maintain germ cell proliferation by mitosis but also to repress the proliferation of steroidogenic cells.

To study the role of *amh* and *amhr2* in ovarian differentiation and early development, two independent methods (E_2 and AI administration) were applied to block testicular development and stimulate ovarian development as demonstrated in our previous studies [10]. In comparison with the control fish, E_2 and AI administration in undifferentiated fish resulted in low levels of *amh* transcripts, and the expression of *amh* remained low in E_2- and AI-induced ovarian tissue. However, *amhr2* transcripts significantly increased in the differentiated ovary in E_2- and AI-induced groups, and ovarian *amhr2* levels were not different from the control testis. These data show that *amh* is specifically expressed during testicular differentiation and that *amhr2* expression is correlated with gonadal development in both sexes in black porgy.

The expressions of *amh* and *amhr2* were detected in the Sertoli cells of spermatogonia and Leydig cells in the testis. Expression of *amh*, but not if *amhr2*, significantly increased in the follicle cells when the oocytes entered vitellogenesis in black porgy. The possible roles of the *amh* gene in the development of vitellogenic oocytes and natural sex change are suggested. Amh expression has been indicated to be important for inhibition of the recruitment of the primordial follicles and the stimulatory effects of follicle-stimulating hormone on the growth of preantral and small antral follicles in the rat [18, 28]. In rats, AMH and AMHR2 were first detectable in the single layer of granulosa cells surrounding large primary follicles, and they are highly expressed in granulosa cells of primarily nonatretic preantral and small antral follicles [18, 28]. In other vertebrates (e.g., in chickens [35], medaka [27], coho salmon [36], and zebrafish [27, 36, 37]), *amh* and *amhr2* transcripts also appeared in the Sertoli cells and Leydig cells of male germ cells and in the follicle cells of female germ cells. In coho salmon, *amh* transcripts significantly increased in the cortical alveolus stage (previtellogenic oocytes) [36] and declined in mature oocytes [37]. In mice, *Amh*-null females and females heterozygous for the *Amh*-null mutation were reported to be fertile, with a normal litter size, but showed a relatively early depletion of their stock of primordial follicles [38]. Moreover,

in the presence of AMH, cultured ovaries contain fewer growing follicles compared to control ovaries [39, 40]. In medaka, loss of function of *amh* and *amhr2* resulted in the suppression of germ cell proliferation during sex differentiation [31]. Nevertheless, excessive proliferation of germ cells was observed in adult medaka fish with the hotei mutation (*amhr2*-deficient) [32]. The finding that *amh* was highly expressed in the premature ovary of black porgy and other vertebrates suggests that the function of *amh* is related to germ cell proliferation and to later ovarian growth. It was demonstrated that high temperatures induced male function and increases of *amh* transcripts in pejerrey fish [41].

During the natural sex change, the values of *amh* expression were variable, with large individual variation during non- to prespawning seasons in testicular tissue. It appeared that there were two groups of *amh* expression in testicular tissue during these periods. Transcripts of *amh* decreased to low levels in January during the spawning period in all 3-yr-old fish. Therefore, we suggest, to our knowledge for the first time, that the fish with high *amh* transcripts during non- to prespawning seasons will remain male during the spawning season; however, the fish with low *amh* transcripts will sexually change to female. To clarify these findings, 2^{+}-yr-old fish were individually labeled, and one of the gonads was surgically removed in the nonspawning season (July–August). The gonadal status in vivo was examined 5 mo later during the spawning season. We showed, to our knowledge for the first time, that the transcription levels of *amh* were dimorphically expressed in the nonspawning season (July and August). Also, in the period from July to August, *amh* transcripts in testicular tissue were correlated with the occurrence of sex change in 3-yr-old fish. It is further suggested that *amh* expression in testicular tissue may be considered as an early marker (5 mo early) of differentiation and of whether natural sex change will occur. We did not find this differentiation in expression of other genes, such as *dmrt1* and *amhr2* (our unpublished data).

Human chorionic gonadotropin regulated *amh* and *amhr2* expression in testicular tissue but not in ovarian tissue. This hCG regulation in the testis was dependent on the gonadal stage. The active testis that underwent hCG stimulation probably entered meiosis (spermatogenesis), which results in decreased *amh* transcripts. In contrast, inactive testicular tissue did not enter spermatogenesis under hCG stimulation, and *amh* transcripts were not deregulated by hCG. Based on the expression of the steroidogenic enzyme genes (*star* and *cyp11a1*) after hCG injection, the responses were differential between active and inactive gonadal tissues. The data suggest that the testicular/ovarian tissues in a single bisexual gonad had differential responses to endogenous stimulation, and they may inhibit each other, as demonstrated by the growth of the ovarian tissue after surgical removal of testicular tissue in previous studies [9].

In summary, the present study demonstrated, to our knowledge for the first time, the possible roles of *amh*/*amhr2* throughout gonadal development in a hermaphrodite fish, the protandrous black porgy, including ovarian and testicular differentiation (0^{+}-yr-old fish), development of a bisexual gonad (1^{+}-yr-old fish), and partial regulation of a later sex change (2^{+}-yr-old fish). Our data suggest that *amh* associates with and is involved in the development of early testicular differentiation and in the development and growth of ovarian tissue during natural sex change.

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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 fish, *Acanthopagrus schlegeli*. Zool Stud 2008; 47:302–317.
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. Lee YH, Wu GC, Du JL, Chang CF. Estradiol-17beta 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.
8. Wu GC, Chang CF. wnt4 is associated with the development of ovarian tissue in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol Reprod 2009; 81:1073–1082.
9. 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.
10. Wu GC, Tomy S, Nakamura M, Chang CF. Dual roles of cyp19a1a in gonadal sex differentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol Reprod 2008; 79:1111–1120.
11. 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.
12. An KW, Lee KY, Yun SG, Choi CY. Molecular characterization of gonadotropin subunits and gonadotropin receptors in black porgy, *Acanthopagrus schlegeli*: effects of estradiol-17β on mRNA expression profiles. Comp Biochem Physiol B Biochem Mol Biol 2009; 152:177–188.
13. Vigier B, Watrin F, Magre S, Tran D, Josso N. Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it in vitro. Development 1987; 100:43–55.
14. Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, et al. Isolation of the bovine and human genes for müllerian-inhibiting substance and expression of the human gene in animal cells. Cell 1986; 45:685–698.
15. Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP, Uilenbroek JT, Karels B, Wilming LG, Meijers JH, Themmen AP, Grootegoed JA. A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the müllerian duct. Development 1994; 120:189–197.
16. di Clemente N, Josso N, Gouédard L, Belville C. Components of the anti-müllerian hormone signaling pathway in gonads. Mol Cell Endocrinol 2003; 211:9–14.
17. Münsterberg A, Lovell-Badge R. Expression of the mouse anti-müllerian hormone gene suggests a role in both male and female sexual differentiation. Development 1991; 113:613–624.
18. Hirobe S, He WW, Lee MM, Donahoe PK. Mullerian-inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 1992; 131:854–862.
19. Smith CA, Smith MJ, Sinclair AH. Gene expression during gonadogenesis in the chicken embryo. Gene 1999; 234:395–402.
20. Western PS, Harry JL, Graves JA, Sinclair AH. Temperature-dependent sex determination in the American alligator: AMH precedes SOX9 expression. Dev Dyn 1999; 216:411–419.
21. Shoemaker C, Ramsey M, Queen J, Crews D. Expression of Sox9, Mis, and Dmrt1 in the gonad of a species with temperature-dependent sex determination. Dev Dyn 2007; 236:1055–1063.
22. Takada S, DiNapoli L, Capel B, Koopman P. Sox8 is expressed at similar

levels in gonads of both sexes during the sex determining period in turtles. Dev Dyn 2004; 231:387–395.
23. Yoshinaga N, Shiraishi E, Yamamoto T, Iguchi T, Abe S, Kitano T. Sexually dimorphic expression of a teleost homologue of Müllerian-inhibiting substance during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun 2004; 322:508–513.
24. Baron D, Houlgatte R, Fostier A, Guiguen Y. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 2005; 73:959–966.
25. Rodríguez-Marí A, Yan YL, Bremiller RA, Wilson C, Cañestro C, Postlethwait JH. Characterization and expression pattern of zebrafish anti-müllerian hormone (Amh) relative to sox9a, sox9b, and cyp19a1a during gonad development. Gene Expr Patterns 2005; 5:655–667.
26. Ijiri S, Kaneko H, Kobayashi T, Wang DS, Sakai F, Paul-Prasanth B, Nakamura M, Nagahama Y. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod 2008; 78:333–341.
27. Klüver N, Pfennig F, Pala I, Storch K, Schlieder M, Froschauer A, Gutzeit HO, Schartl M. Differential expression of anti-müllerian hormone (amh) and anti-müllerian hormone receptor type II (amhrII) in the teleost medaka. Dev Dyn 2007; 236:271–281.
28. Baarends WM, Hoogerbrugge JW, Post M, Visser JA, De Rooij DG, Parvinen M, Themmen AP, Grootegoed JA. Anti-müllerian hormone and anti-müllerian hormone type II receptor messenger ribonucleic acid expression during postnatal testis development and in the adult testis of the rat. Endocrinology 1995; 136:5614–5622.
29. Lee MM, Seah CC, Masiakos PT, Sottas CM, Preffer FI, Donahoe PK, Maclaughlin DT, Hardy MP. Müllerian-inhibiting substance type II receptor expression and function in purified rat Leydig cells. Endocrinology 1999; 140:2819–2827.
30. Behringer RR, Finegold MJ, Cate RL. Müllerian-inhibiting substance function during mammalian sexual development. Cell 1994; 79:415–425.
31. Shiraishi E, Yoshinaga N, Miura T, Yokoi H, Wakamatsu Y, Abe S, Kitano T. Mullerian-inhibiting substance is required for germ cell proliferation during early gonadal differentiation in medaka (*Oryzias latipes*). Endocrinology 2008; 149:1813–1819.
32. Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, Shimizu N, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H. The hotei mutation of medaka in the anti-mullerian hormone receptor causes the dysregulation of germ cell and sexual development. Proc Natl Acad Sci U S A 2007; 104: 9691–9696.
33. Pask AJ, Whitworth DJ, Mao CA, Wei KJ, Sankovic N, Graves JA, Shaw G, Renfree MB, Behringer RR. Marsupial anti-mullerian hormone gene structure, regulatory elements, and expression. Biol Reprod 2004; 70:160–167.
34. Maugars G, Schmitz M. Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. Gen Comp Endocrinol 2008; 159:178–187.
35. Oréal E, Mazaud S, Picard JY, Magre S, Carré-Eusèbe D. Different patterns of anti-müllerian hormone expression, as related to DMRT1, SF-1, WT1, GATA-4, Wnt-4, and Lhx9 expression, in the chick differentiating gonads. Dev Dyn 2002; 225:221–232.
36. Luckenbach JA, Iliev DB, Goetz FW, Swanson P. Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, *Oncorhynchus kisutch*. Reprod Biol Endocrinol 2008; 6:2.
37. von Hofsten J, Larsson A, Olsson PE. Novel steroidogenic factor-1 homolog (ff1d) is coexpressed with anti-mullerian hormone (AMH) in zebrafish. Dev Dyn 2005; 233:595–604.
38. Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-müllerian hormone in the mouse ovary. Endocrinology 1999; 140: 5789–5796.
39. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 2002; 143:1076–1084.
40. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 2001; 142:4891–4899.
41. Fernandino JI, Hattori RS, Kimura H, Strüssmann CA, Somoza GM. Expression profile and estrogenic regulation of anti-müllerian hormone during gonadal development in pejerrey *Odontesthes bonariensis*, a teleost fish with strong temperature-dependent sex determination. Dev Dyn 2008; 237:3192–3199.