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Testicular *dmrt1* Is Involved in the Sexual Fate of the Ovotestis in the Protandrous Black Porgy[1]

Guan-Chung Wu,[3] Po-Chia Chiu,[4] Chien-Ju Lin,[4] Ying-Syuan Lyu,[4] Dan-Suei Lan,[4] and Ching-Fong Chang[2,3,4]

[3]*Center of Excellence for Marine Bioenvironmental and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan*
[4]*Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan*

ABSTRACT

In hermaphroditic fish, the ovotestis can respond to external stimuli so that only one type of gonadal tissue (either ovarian or testicular tissue) will remain reproductively active and the other will recede to a rudimentary stage. However, the molecular mechanism for sexual fate determination is still poorly understood in hermaphroditic fish. In the present study, we examined whether sexual fate determination with respect to testis development is due to differential expression of *dmrt1*. Expression of *dmrt1* was limited to the spermatogonia-surrounding cells (Sertoli cells) throughout testis development. Testicular *dmrt1* was differentially expressed in fish (black porgy [*Acanthopagrus schlegeli* Bleeker]) depending on if fish were destined to be female or male. Expression of *dmrt1* in Sertoli cells did not require germ cell factors with busulfan treatment. To examine the role of *dmrt1*, we used virus-based RNA interference. Deficiency of *dmrt1* resulted in a reduced number of germ cells in the testis and stimulated a male-to-female sex change. Higher serum luteinizing hormone levels were detected in 2^+- to 3-yr-old male fish as compared to sex-changing female fish. Furthermore, we showed that fish treated in vivo with gonadotropin-releasing hormone (Gnrh) and fish treated in vitro with gonadotropin (Gth) had higher *dmrt1* expression in the testis, suggesting that these endocrine factors may affect the male-to-female sex change. Therefore, our data suggest that *dmrt1* plays a key role in initial testis differentiation and in later maintenance of male development. We show, to our knowledge for the first time, the functions of *dmrt1* in hermaphroditic fish, which indicate that male-phase maintenance may be regulated by the brain-pituitary-gonadal axis via the Gnrh-Gth-Dmrt1 axis.

gonadal function, hermaphroditism, ovary, sex change, sex determination, sex differentiation, testis

INTRODUCTION

Sex determination is an ancient and universal feature in animals. All animals fall into one of two classifications that define their sexual fate: gonochores, which have fixed sexes, and hermaphrodites, which can have both sexes during their life span. Sex change in hermaphrodites occurs in a variety of animals, including annelids, echinoderms, crustaceans, mollusks, and fish [1]. The capacity for sex change is lost from amphibians to mammals. However, most of our knowledge regarding sex differentiation is from studies of gonochoristic species. How the initial and terminal sexes of sequential hermaphroditic fish are determined is still unknown.

The black porgy (*Acanthopagrus schlegeli* Bleeker) is a protandrous hermaphroditic fish with a striking life cycle (Fig. 1). The fish are functional males for the first 2 yr of life, but approximately 50% of them transform into females during the third year [2–6]. The ovotestis (comprising the testis and ovary, which are separated by connective tissue) is first observed in 4- to 5-mo-old fish [4]. The fish will be functional males during the first and second spawning period, and 50% of the fish will change to females in the third spawning period. These findings suggest the existence of a switch that can alternate between the testis and ovary in response to the external stimuli. Therefore, the black porgy is a unique model in which to study initial gonadal differentiation, sexual fate determination, and terminal natural sex change.

Increased plasma estradiol (E2) levels correlate with the natural sex change in the third reproductive cycle [2, 3], which can be blocked by long-term administration of aromatase inhibitor [7]. However, vitellogenic oocytes were infrequently observed following long-term treatment with E2 in 0^+- and 1^+-yr-old fish [8–10], even though ovarian tissue containing primary oocytes was still the main portion of the ovotestis. According to histological observations, only one type of gonadal tissue (either testicular or ovarian tissue) will remain reproductively active, and the other will recede to a rudimentary stage [4]. It is thought that the testicular tissue acts to inhibit ovarian development. Instead of the natural regression of the ovarian tissue in the male phase in 2-yr-old fish, we conducted a surgical elimination of the testicular tissue in 1^+-yr-old fish. This approach resulted in the development of ovarian tissue and the female phase in 100% of the 1^+-yr-fish [9–12]. The expression of female-related genes (*wnt4*, *foxl2*, and *cyp19a1a*) and steroidogenesis-related genes (*nr5a1a*, *nr0b1*, *star*, *cyp11a1*, and *hsd3b1*) is consistently high in castrated 1^+-yr-old fish and 2^+- to 3-yr-old fish undergoing the natural sex change [9, 10]. Therefore, we suggest that maintenance of the male phase inhibits the sex-change process in the black porgy.

In mammals, both formation of the testis and subsequent male development are initiated by the sex-determining *SRY* gene on the Y chromosome [13, 14]. By contrast, the doublesex and mab-3-related transcription factor 1 (DMRT1) is a gonad-specific transcription factor related to the invertebrate sexual regulator. *DMRT1* [15] and its paralogues are candidates for sex-determining genes in nonmammalian vertebrates, including the chicken Z-linked *DMRT1* [16], the frog W-linked *DM-W* [17–19], and the medaka Y-linked *dmy*/*dmrt1by* [20–22]. Furthermore, *DMRT1* has been suggested to be an important transcriptional regulator of male differentiation that is required for testis development in vertebrates (mammals

[1]Partially supported by the National Science Council and Ministry of Education (Taiwan).
[2]Correspondence: E-mail: b0044@mail.ntou.edu.tw

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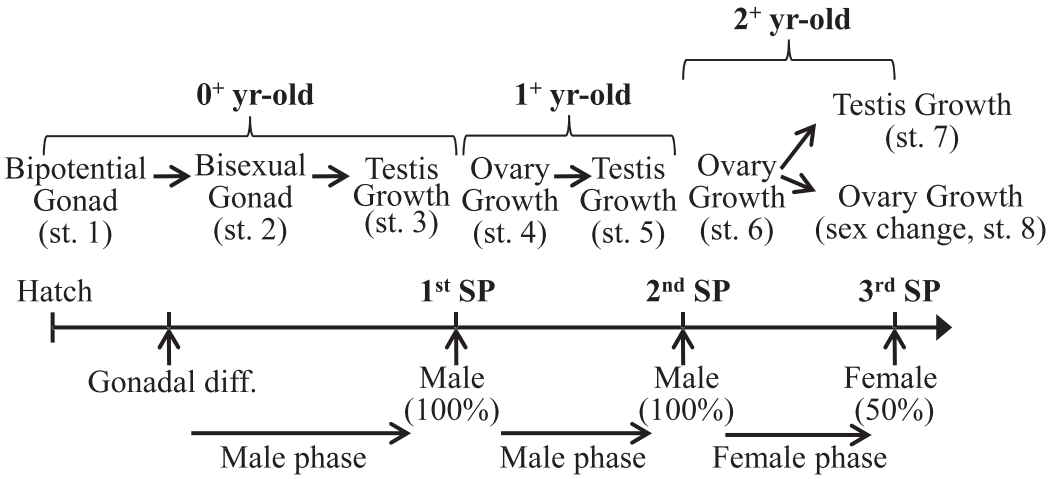

FIG. 1. Reproductive and life cycle in relation to gonadal development in the black porgy. Stage of gonadal development in 0^+- to 3-yr-old fish is shown. Stage 1, undifferentiated gonad; stage 2, differentiated ovotestis; stage 3, testis development with a tiny ovary in the bisexual gonad; stage 4, ovary development with a degenerated testis in the bisexual gonad; stage 5, testis growth with a degenerated ovary in the male gonad; stage 6, ovary growth with a degenerated testis in the bisexual gonad; stage 7, testis growth with a degenerated ovary in the male gonad; stage 8, ovary growth and vitellogenesis (sex change) with a regressed testis in the female gonad. diff., differentiation; SP, spawning period; st., stage.

[23], birds [24, 25], reptiles [26], amphibians [27], and teleosts [28–31]). However, different *DMRT1* expression patterns have been reported in different vertebrate species. In mice [32] and chickens [33], *DMRT1* expression has been observed both in Sertoli cells and germ cells. In humans [34] and lizards [35], *DMRT1* is expressed initially in Sertoli cells and later in germ cells. Even in fish, the cell types with *dmrt1* expression are highly variable among species [36]. Expression of *dmrt1* has been reported in Sertoli cells (medaka [29] and tilapia [31]), in germ cells (grouper [30] and zebrafish [37]), and in both (platyfish [38]).

In mice, *Dmrt1* is required in Sertoli cells for postnatal testis differentiation, germ line maintenance, and meiotic progression [39]. *Dmrt1* is also required for the radial migration of the germ cells to the periphery of the seminiferous tubule, where the spermatogenic niche is formed, and for mitotic reactivation [39]. Furthermore, the gene expression profile in the testes of *Dmrt1*-mutant mice shows disruptions in several important testicular signaling pathways (*Gdnf*, retinoic acid, *Tgf*, and *Fsh*) and the expression of pluripotency markers (*Stella*, *Dppa3*, and *Pgc7*) [40]. Although the expression of *DMRT1* in the Sertoli and germ cells may vary in vertebrates, recent results in mice indicate that DMRT1 is important for maintaining the germ line cell niche and the pluripotency of germ cell precursors [40]. Furthermore, in chickens, the reduction of *DMRT1* expression resulted in the feminization of the embryonic gonads in genetic males [16]. In tilapia, overexpression of *dmrt1* in genetic females resulted in various degrees of follicular degeneration and partial or complete sex reversal [41].

Sex change in sequential hermaphroditic fish is initiated through the changes in neural signaling and sex steroids, but we are only beginning to understand the specific mechanism by which this occurs through the brain-pituitary-gonadal axis. Few studies in the sequential hermaphroditic fish support a role of a gonadotropin-releasing hormone (Gnrh)-gonadotropin (Gth)-mediated sex change. The numbers of Gnrh neurons are greater in the male phase than in the female phase in both the protogynous wrasse [42, 43] and protandrous anemonefish [44]. Administration of Gnrh with a dopamine receptor antagonist and human chorionic gonadotropin (hCG) in the female wrasse stimulated a female-to-male sex change [45, 46]. Furthermore, in mice, *Dmrt1* was stimulated by GTH administration in Sertoli cells [47]. Therefore, the role of *dmrt1* expression and the association with Gth in the sexual fate of the protandrous black porgy are of interest. Thus, we hypothesized that Dmrt1 and Gth are involved in sexual determination and are required for testis development and male maintenance in the black porgy.

We investigated the mechanisms of sex development at the molecular, cellular, and organismal levels using the following experimental approaches: E2 to inhibit *dmrt1* expression, busulfan to disrupt germ cells, unilateral orchidectomy, *dmrt1* knockdown, and the association of Gth and *dmrt1* with the sex change. Although many studies have focused on *dmrt1* function in relation to sex determination and gonadal differentiation in fish, we are the first, to our knowledge, to elucidate the function of *dmrt1* during early gonadal differentiation and subsequent testis growth. We have successfully developed a technique for the delivery of shRNA to disrupt the function of *dmrt1*. Our results demonstrate that *dmrt1* is important not only for testis differentiation but also for sexual fate determination and the natural sex change. The Gnrh-Gth axis may mediate the *dmrt1* function for male maintenance.

MATERIALS AND METHODS

Experimental Fish

Black porgy at different ages (0^+ to 3 yr) were used in the experiments. The gonadal development is summarized in Figure 1. The experimental fish were acclimated to the pond environment at the National Taiwan Ocean University culture station in seawater and with a natural lighting system. Water temperatures ranged from 19°C to 26°C in natural condition. The fish were fed with a 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.

Experimental Design

For each experiment, the stage of gonadal development is described in Figure 1.

Experiment 1: Localization and expression profile of dmrt1 *during gonadal differentiation and testis development.* Fish (age, 0^+ to 1^+ yr) were collected during gonadal differentiation (stages 1 and 2) (Fig. 1) and testis development (stage 3) (Fig. 1) from May 2008 to April 2009 for genetic analysis, histology,

immunohistochemical (IHC) staining, and immunofluorescence (IF) staining. We first established Dmrt1 (GenBank accession no. AY323953) antiserum to analyze the Dmrt1 expression profile and Vasa (GenBank accession no. DQ399799) antiserum to identify early germ cells. Furthermore, in situ hybridization of *dmrt1* was used to confirm IHC and IF with Dmrt1 antiserum.

Experiment 2: dmrt1 *expression during the natural sex change*. Fish (age, 2^+ to 3 yr; n = 8 per sample point) were collected from May 2007 to February 2008 for genetic analysis, histology, and IF staining. Of these fish, 50% underwent a sex change in the third reproductive period. To further examine the *dmrt1* profile during a natural sex change, we first performed unilateral orchidectomies to collect one of the bilateral gonads at stage 6 (Fig. 1), while the testis was still regressed (5–6 mo before the third spawning period), in 2^+-yr-old fish (n = 22) and then chip-labeled the fish to trace their sexual fate during the spawning period of the third reproductive cycle (3-yr-old fish of either sex).

Experiment 3: Effects of estrogen at different gonadal developmental stages. To study the effects of E2 on *dmrt1* expression, we administered E2 (2 μg/g body wt, two intraperitoneal injections in 5 days; n = 8 per group for the E2 and control groups) at stage 3 (Fig. 1). To further clarify the effects of E2 on gonadal development, fish were fed a diet containing E2 (6 mg/kg feed) at the undifferentiated gonadal stage (age, 3 mo) and the differentiated gonadal stage (age, 5 mo) for 3 mo, followed by replacement of the E2 diet with control feed until the first spawning season. Samples (n = 8 fish/group) were collected monthly for genetic analysis and histology.

Experiment 4: dmrt1 *knockdown in juvenile fish*. To examine the role of *dmrt1* in the initial testis differentiation and later development, we performed a knockdown analysis of *dmrt1* in the testis using RNA interference (RNAi). We used a lentiviral approach to deliver shRNA designed to knock down *dmrt1* expression. The vector contains the U6 promoter to achieve sufficient expression of the shRNA and the vesicular stomatitis virus G (VSV-G) to achieve a high rate of infection. We examined the effects of shRNA on the *dmrt1* transcripts during the initial testis differentiation and in testis development of 0^+-yr-old fish. The control fish were infected with a vector encoding shRNA against the luciferase gene. The shRNA was injected three times (3.7×10^6 colony-forming units each time per fish) at stage 1 (before gonadal differentiation) or at stage 2 (when the spermatogonia have already entered mitosis and are widely distributed) (Fig. 1). Fish were collected at stage 3 (age, 6 mo) (Fig. 1) and at the spawning season (age, 11 mo) for genetic analysis, histology, and IHC staining. To confirm that virus was restricted to the injected gonad during the course of the experiment, the extracted DNA was analyzed by PCR for the zebrafish U6 promoter. The oligonucleotides 5′-TCCATATTGCTGTTTTAGTGCGTGG-3′ and 5′-TGCGCAGGGGCCATGCT-3′ complementary to the zebrafish U6 promoter were used as the primer.

Experiment 5: Effects of germ cell loss during gonadal development. Treatment with busulfan was performed to inhibit germ cell proliferation. Fish were fed a busulfan diet (20 mg/kg feed) from the undifferentiated gonad (stage 1; age, 2 mo) to stage 3 (age, 8 mo) (Fig. 1) for 6 mo, after which busulfan administration was replaced by the control diet until the first spawning season. Samples (n = 8 per group) were collected monthly for genetic analysis, histology, IHC, and IF staining.

Experiment 6: Effects of Gth on dmrt1 *expression*. To investigate the relationship between serum luteinizing hormone (Lh) levels and the natural sex change, the serum Lh profiles during the natural sex change were studied in 2^+-yr-old fish. Blood samples for Lh radioimmunoassay (RIA) were collected continuously and regularly at 1-wk intervals from April to June in 2^+-yr-old male fish (n = 13; each individual was chip-labeled) (see the gonad at stage 6 in Fig. 1). The sexual status of each fish during the third spawning period was determined by biopsy, and the fish were classified as either males or sex-changed females.

To determine the effects of Gth on *dmrt1* expression, fish at gonadal stage 6 (n = 8 per group for the treated and control groups) (Fig. 1) were injected with a luteinizing hormone-releasing hormone (LHRH) analog (15 mg/kg body wt; Sigma) on Days 1 and 5. Gonads (testicular and ovarian tissues) were collected 24 h after the second injection, and analysis of *dmrt1* expression was performed. To further clarify the effects of Gth on *dmrt1* expression, we gave hCG through an in vitro organ culture system. Gonads were collected at stage 5 (Fig. 1), and the ovarian tissue was removed from the ovotestis. The hCG (high and low doses, 2 and 2×10^{-3} IU/ml culture medium, respectively; Sigma) was administered for a 24-h period, and testicular tissues was collected at 12 h, 24 h, Day 3, and Day 6 for *dmrt1* analysis.

Plasmid Construction

For the *dmrt1* knockdown vectors, we first replaced the hybrid cytomegalovirus-zebrafish U6 promoter [48] in RNAi-Ready pSIREN-retroQ (Clontech). Next, the double-stranded oligonucleotides corresponding to 5′-GATTCGAAGTGCTCCCGCTGTAGGAATTTCAAGAGAATTCCTACAGCGGGAGCACTTCTTTTTTGAATT-3′ were inserted into the *Eco*RI and *Bam*HI sites of the resulting vector to generate sh*dmrt1*. The control vector contained specific double-stranded oligonucleotides for the luciferase gene (Clontech).

Virus Production

Virus production was performed according to the manufacturer's protocol (Clontech). The VSV-G glycoprotein pseudotyped retroviral vector has very high titers and efficient gene transfer in fish. Therefore, for producing recombinant virus, we first cotransfected pVSV-G (Clontech) and pSIREN-retroQ-*shdmrt1* or pSIREN-retroQ-*sh*Luciferase into GP2-293 packing cell lines to produce virus and used HeLa cells to check infectious ability.

In Situ Hybridization

We used antisense RNA probes for *dmrt1* (nucleotides 272–911), and in situ hybridization of tissue section was performed as described previously [11].

Antiserum Production

We used the synthesized peptide-ovalbumin conjugation to immunize antiserum. The *dmrt1* antiserum was produced in white rabbits immunized against a peptide fragment of black porgy Dmrt1 (CEASSETPNFTVSSIID). The Vasa antiserum was produced in guinea pigs immunized against a peptide fragment of black porgy Vasa (IHGDREQRREREQALAD and STDNRKGGSFQENGGQ). The antisera were prepared by Yao-Hong Biotechnology, Inc. Extracts of Dmrt1- and Vasa-expressing HEK293 cells were used to confirm the specificity of the antiserum by Western blot analysis as described by Wu and Chang [11]. Immunoblotting was performed with preabsorbed antibodies at 4°C overnight, with the blocking buffer containing 1.5% nonfat dry milk. After washing, nitrocellulose membranes were incubated with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody (Amersham) at 4°C overnight. Finally, the ECL Plus Western Blotting Detection System (Amersham) was used to detect the protein staining. A specific and single protein band corresponding to black porgy Dmrt1 or to Vasa (according to the molecular weight) was detected.

Histological Analysis, IF Staining, and IHC Staining

Hematoxylin-and-eosin staining and IHC staining were performed as described previously [10]. IF experiments were performed on a gonad fixed with 4% paraformaldehyde in PBS. Antiproliferating cell nuclear antigen (Pcna) antiserum (Santa Cruz Biotechnology) diluted 1:200 was used as described by Wu and Chang [11]. Anti-Dmrt1 and anti-Vasa antisera were used at a dilution of 1:3000.

DNA and RNA Analysis

Gonads were collected and homogenized in TRIzol reagent (Invitrogen). This homogenate was used for both RNA and DNA analysis. Extractions of genomic DNA, total RNA, and first-strand cDNA were performed according to the manufacturer's protocol. Total RNA (1 μg) extracted from gonad of the representative fish was reverse transcribed to the first-strand cDNA using Superscript II (Invitrogen) with the oligo$(dT)_{12–18}$ primers. This first-strand cDNA was used for the PCR and quantitative real-time PCR (Q-PCR) analyses. Specific primers for *dmrt1*—namely, *figla* (factor in germ line alpha; GenBank accession no. EU496494), *amh* (antimüllerian hormone; GenBank accession no. GU256046), and *amhr2* (antimüllerian hormone type II receptor; GenBank accession no. GU256045)—were as previously described [10, 12, 28]. Q-PCR was performed as previously described [9]. All samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; GenBank accession no. DQ399798), and control values were defined as one.

Analysis of Lh Levels Using RIA

The Lh levels were measured by a homologous RIA using purified black porgy Lh as a standard and anti-black porgy Lh serum as antibody [8, 49].

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. Student *t*-test was also conducted to determine significant differences ($P < 0.05$) between treatments.

RESULTS

dmrt1 *Is Localized in the Somatic Cells Surrounding the Spermatogonia and Is Expressed Throughout Testis Development*

Expression of *dmrt1* was found in the somatic cells (Sertoli cells) surrounding the spermatogonia (Fig. 2a). We next examined the localization of Dmrt1 by IHC staining using a specific anti-Dmrt1 antibody. The specificity of the antibody to Dmrt1 was confirmed by Western blot analysis using ovotestis extracts and recombinant Dmrt1 (Fig. 2b). IHC staining confirmed that Dmrt1 was only expressed in the Sertoli cells (Fig. 2c). Furthermore, we used anti-Vasa antibody, a germ cell marker, to confirm the localization of Dmrt1 in Sertoli cells by IF staining (Fig. 2d). According to our IHC results, Dmrt1 was expressed in the Sertoli cells of the testis, and the expression of Dmrt1 was increased in the differentiated testis (stages 1 and 2) (Fig. 2, e and f) and later in testis development (stage 3) (Fig. 2g). IF staining revealed that the expression of Dmrt1 in Sertoli cells was high in the developing gonad (stage 3) but greatly decreased in the mature testis (Fig. 2, h and i), and Dmrt1 remained at low levels in the regressed testis after the spawning period (stage 4) (Fig. 2j).

Disappearance of dmrt1 *in the Testis Disrupts Male Development and Is Associated with the Sex Change*

To obtain male-fated and female-fated fish, we removed one of the bilateral ovotestes in 2^{+}-yr-old fish. Q-PCR showed a sexually dimorphic expression pattern of *dmrt1* in the gonad with high levels in male-fated individuals and consistently high expression throughout testis development (stages 6 and 7) (Fig. 3, a and b) and low levels in female-fated individuals (stage 6) (Fig. 3b). Q-PCR showed no difference in testicular *amh* between male-fated and female-fated fish; *amh* expression was maintained at low levels (stage 6) (Fig. 3b). Differential expression of *dmrt1* was observed 5–6 mo before the sex change occurred. IF confirmed robust Dmrt1 expression in male-fated individuals (stages 6 and 7) (Fig. 3, c–f), whereas Dmrt1 expression was lost in the Sertoli cells of female-fated individuals (stages 6 and 8) (Fig. 3, g–i). Finally, we observed a complete loss of germ cells in the regressed testis after the natural sex change (Fig. 3j).

dmrt1 *in the Sertoli Cells Is Required for Testis Differentiation*

After E2 injection (short-term treatment) at stage 3, we found that Dmrt1 expression was decreased compared to the control fish (Fig. 4, a and b). Q-PCR confirmed that *dmrt1* expression was significantly decreased in the E2-treated group compared to controls ($P < 0.05$). Thus, to evaluate the correlation between gonadal differentiation/development and *dmrt1* expression, we used long-term E2 administration to induce testicular regression and ovarian development at different stages before and after ovarian cavity formation. At the end of the experiment, control fish showed the mature male phase with an ovotestis during the first spawning period (Fig. 4, c and d). After 3 mo of E2 treatment, testicular tissue regressed and ovarian tissue developed in both groups (Fig. 4, e and g). After E2 termination, oogenesis (oogonia/primary oocytes) was observed in the area of the ''regenerating testis'' when E2 administration was given at the undifferentiated gonadal stage (Fig. 4f). By contrast, only a few degenerating oocytes were found in the ''regenerating testis'' when E2 administration was given at the gonadal differentiation (Fig. 4h). Q-PCR revealed that gonadal *dmrt1* expression was decreased by 6.7-fold ($P < 0.05$) in E2-treated fish compared to controls. Furthermore, *dmrt1* was increased in the E2-terminated gonad (with a regenerating testis), which was not significantly different from control male testicular tissue.

Knockdown of dmrt1 *in Fish*

After virus was injected (i.p.) into fish three times at 1-wk intervals, the fish all survived, with no effects on the visceral organs. We confirmed the effects of shRNA on the *dmrt1*expression in juvenile fish. We performed PCR for the vector sequence to confirm that the packaged virus infected the cells of the gonad and delivered the gene (Fig. 5a). Some fish retained endogenous levels of *dmrt1*, and others showed a slight or significant decrease in *dmrt1* transcripts (fish 3 and 7 in Fig. 5b). Differences in infection efficiency caused differences in development of the gonadal tissue, even between the bilateral gonads in one individual (a normal gonad on one side and an abnormal gonad without formation of an ovarian cavity on the other side) (Fig. 5c).

dmrt1 *Deficiency in the Undifferentiated Testis Disrupted Testicular Differentiation and Stimulated Ovarian Development*

We used shRNA to knock down *dmrt1* expression in the undifferentiated gonad. All control fish (n = 10) had normal male-phase gonads. Two months after virus injection and when spermatogonia enter mitosis in controls (Fig. 5d), 8 of 10 injected fish had normal gonadal morphology, and the other two had abnormal or sex-changed gonads. We observed a mono-ovary (not an ovotestis, without connective tissue to separate the ovarian and testicular tissues), and putative female germ cells were widely distributed throughout the gonad (Fig. 5, e and f). We also found that *figla* (an ovarian marker) expression was increased in the gonads of the individuals with lower *dmrt1* expression (fish 3 and 7 in Fig. 5b) compared to individuals with normal *dmrt1* expression (control and fish 2 in Fig. 5b).

dmrt1 *Deficiency in the Differentiated Testis Leads to Testis Regression and Further Ovarian Development*

To evaluate testis development in the *dmrt1* knockdown fish, we histologically examined the testes of control and injected fish at the spawning stage (age, 11 mo). Control fish had normal ovotestes, containing a large portion of mature testis and a tiny portion of regressed ovary separated by connective tissue (Fig. 5g). All control fish (n = 32) had normal male-phase gonads. Most shRNA-injected fish (29 of 41) also had a normal ovotestis. However, 12 of 41 transgenic fish had an abnormal gonad, with a significantly reduced number of germ cells, and displayed two types of morphological differences. First, 9 of 12 abnormal gonads had a highly degenerated testis on one side and a normal testis on the other side (Fig. 5h). Second, 3 of 12 abnormal individuals had a highly degenerated testis and another fully developed ovary (in the primary oocyte stage) in both gonads (Fig. 5, i and k). To confirm this loss of male germ cells in the degenerated testis, no Vasa-positive cells were found in the degenerated testis of the *dmrt1*-deficient fish (Fig. 5, j and k).

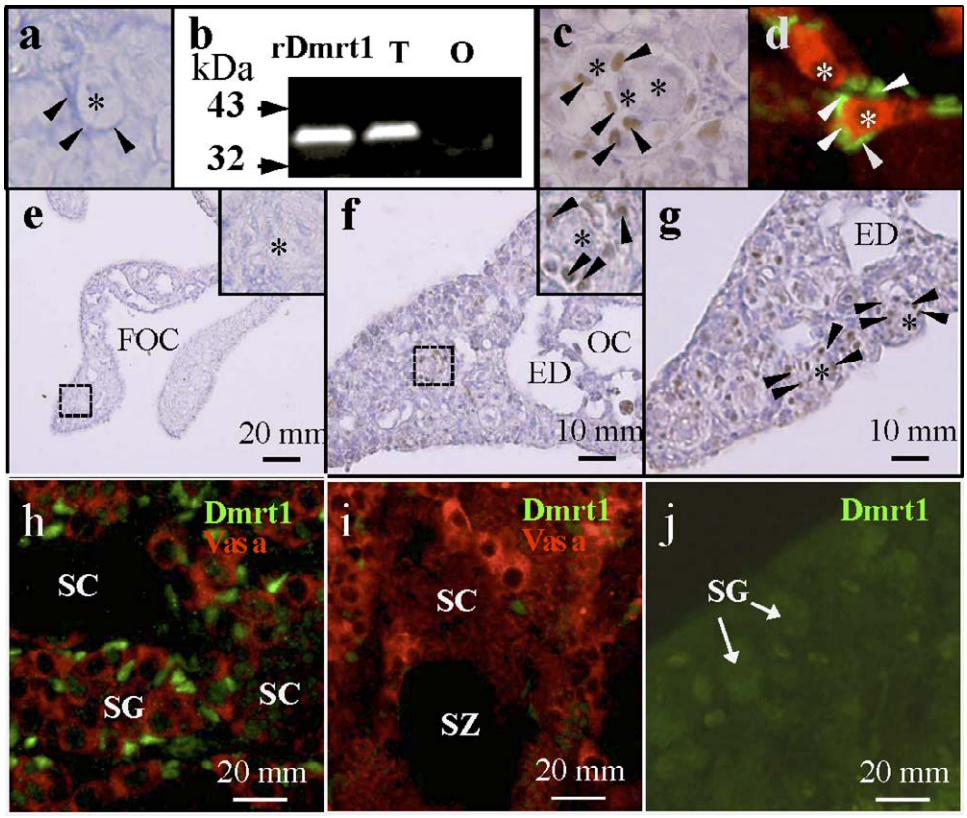

FIG. 2. Expression of *dmrt1* in the somatic cells surrounding the spermatogonia during the male reproductive cycle. Localization and expression of *dmrt1* are shown. **a**) In situ hybridization analysis of *dmrt1* in testis. **b**) Western blot analysis using a Dmrt1 antibody. HEK293 cells were transiently transfected with pcDNA-*dmrt1*. Extracts from HEK293 cells, testis, and ovary were examined by immunoblotting with a Dmrt1 antibody followed by a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. Dmrt1 was only detected in the testis. **c**) IHC analysis of the testis with a Dmrt1 antibody. **d**) IF analysis of the testis using Dmrt1 and Vasa antibodies. **e**–**j**) IHC (**e**–**g**) and IF (**h**–**j**) staining of Dmrt1 at different developmental stages, including undifferentiated gonad (stage 1; **e**), differentiating testis (stages 1–2; **f**), differentiated testis (stage 2; **g**), developing testis (stage 3; **h**), mature testis (**i**), and inactive testis during the postspawning period (stage 4; **j**). Arrowhead indicates Sertoli cell; asterisk indicates spermatogonia. ED, efferent duct; FOC, formation of ovarian cavity; SC, spermatocyte; SG, spermatogonia; SZ, spermatozoa.

dmrt1 *Expression in Sertoli Cells Does Not Require Germ Cells*

To determine the role of germ cells in somatic cell differentiation, treatment with busulfan was performed in the undifferentiated gonad (age, 2–8 mo) to inhibit germ cell proliferation. We found that busulfan prevented the germ cells from entering mitosis in the testis (Fig. 6a). By contrast, Q-PCR revealed that several testis-related genes (*dmrt1*, *amh*, and *amhr2*) had similar expression levels in both the control and busulfan-treated groups during early testis development (Fig. 6a). At the time of gonadal differentiation, the number of spermatogonia was significantly increased in the control testis, and Pcna was highly expressed in the spermatogonia (Fig. 6b). In busulfan-treated fish, the number of germ cells was low in both the testis (Fig. 6, c and e) and the ovary (data not shown), whereas robust Pcna expression was found in the somatic cells (Fig. 6c). Dmrt1 was expressed in the Sertoli cells in both the control and busulfan-treated groups after testis differentiation, but the germ cell number was significantly reduced in the busulfan-treated fish (Fig. 6, d and e).

Expression of dmrt1 *in Testis Requires Gth*

Significantly lower levels of serum Lh were detected in the male-to-female sex change fish during the post- and non-spawning periods (from April to June) compared to the 2^{+}- to 3 yr-old fish that remained in the male phase (male-to-male) (Fig. 7a). The expression of *dmrt1* was stimulated in the testis after LHRH analog treatment compared to controls (Fig. 7b). To further assess how Gth affects *dmrt1* expression, we utilized an in vitro tissue culture assay. The expression of *dmrt1* was decreased in the control testis during the culture period (Fig. 7c), whereas hCG treatment up-regulated (i.e., rescued) the expression of *dmrt1* in the testis compared to controls (Fig. 7c).

DISCUSSION

The present study was undertaken to systemically characterize the expression and function of the *dmrt1* gene in an animal model. Using shRNA, we successfully disrupted the function of *dmrt1* in vivo. We examined the role of *dmrt1* in gonadal differentiation and testis development using stage-specific gene knockdown. Our data show that *dmrt1* deficiency results in testicular regression and sex change.

Sertoli Cell Expression of dmrt1 *Is Required for Testis Differentiation*

Immunohistochemical analysis with an anti-Dmrt1 antibody revealed that Dmrt1 is localized to somatic cells surrounding germ cells in the black porgy during gonadal differentiation. This is similar to other vertebrates and shows that *dmrt1* is expressed in a variety of cell types, including somatic and/or germ cells [27, 29–35, 37–38]. These results suggest that *dmrt1* function may vary among species.

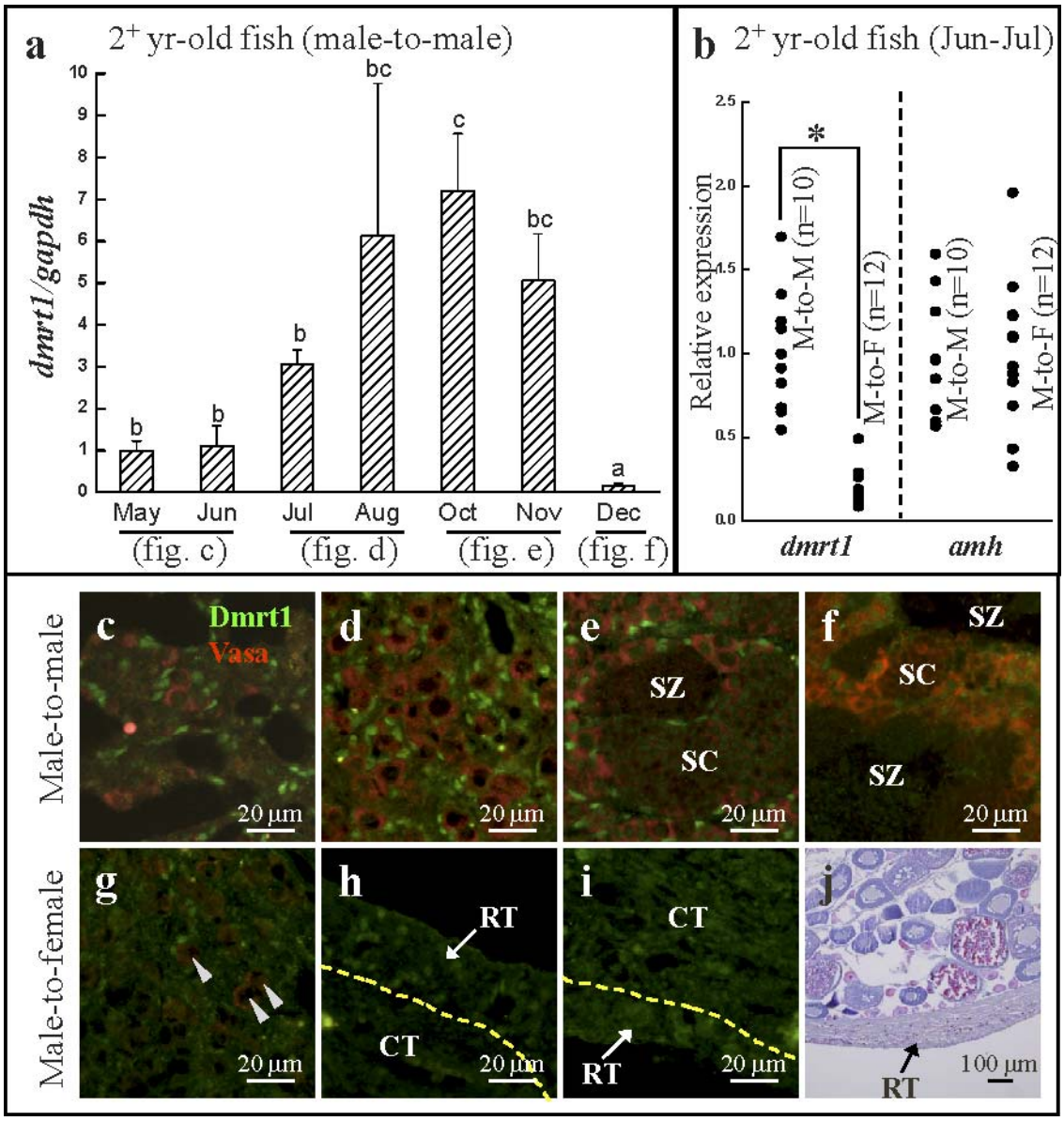

FIG. 3. Expression profile of *dmrt1* during the natural sex change in 2^+- to 3-yr-old fish. Expression of *dmrt1* in the testis of male-to-male fish was measured by Q-PCR from May to December (**a**). Superscript letters indicate one-way ANOVA and a Student-Newman-Keuls multiple test ($P < 0.05$). Expression of *dmrt1* and *amh* in the testis (stage 6; collected from the post- and nonspawning season) of male-to-male fish and male-to-female sex change fish was measured by Q-PCR (**b**). Asterisk indicates a Student *t*-test ($P < 0.05$). IF staining for Dmrt1 (green) and Vasa (red) in the gonads of male-to-male fish (**c**–**f**) and male-to-female sex change fish (**g**–**i**) and hematoxylin-and-eosin staining in the gonad of male-to-female sex change fish (**j**) at the differentiated period are also shown. Arrowhead indicates spermatogonia. CT, connective tissue; RT, regressed testis; SC, spermatocyte; SZ, spermatozoa.

We then investigated whether exogenous estrogen could reverse testis differentiation/development by suppressing *dmrt1* expression in Sertoli cells (Fig. 4). We utilized RNAi to knock down *dmrt1* expression in the undifferentiated gonads and obtained abnormal gonads (with a mono-ovary) with widespread female germ cells. Q-PCR showed that gonadal *amh* (a testicular marker) expression was decreased but that *figla* (an ovarian marker) expression was increased in the fish with lower *dmrt1* expression as compared to the individuals expressing normal levels of *dmrt1* (data not shown). This shows that *dmrt1* deficiency disrupts testis differentiation and also partially stimulates sex reversal. Taken together with observations from other species [16, 41], these data suggest that *dmrt1* plays a role in testis differentiation.

Testis Development Is Mediated by Sertoli Cell dmrt1 *Through the Regulation of Spermatogonial Proliferation*

To evaluate the precise function of *dmrt1*, we knocked down *dmrt1* expression in the differentiated gonad and found a reduced number of spermatogonia in the testis and a complete loss of germ cells during the spawning period. In mice, a conditional deletion of *Dmrt1* revealed that it is required for postnatal Sertoli cell differentiation and for germ line maintenance and meiotic progression [39]. Loss of *Dmrt1* in adult spermatogonia caused a precocious exit from the spermatogonial program and entry into meiosis in mice [50]. These results demonstrate that *dmrt1* has different functions in Sertoli cells and germ cells with regard to testis development. By contrast, grouper *dmrt1* is expressed only in spermatogonia

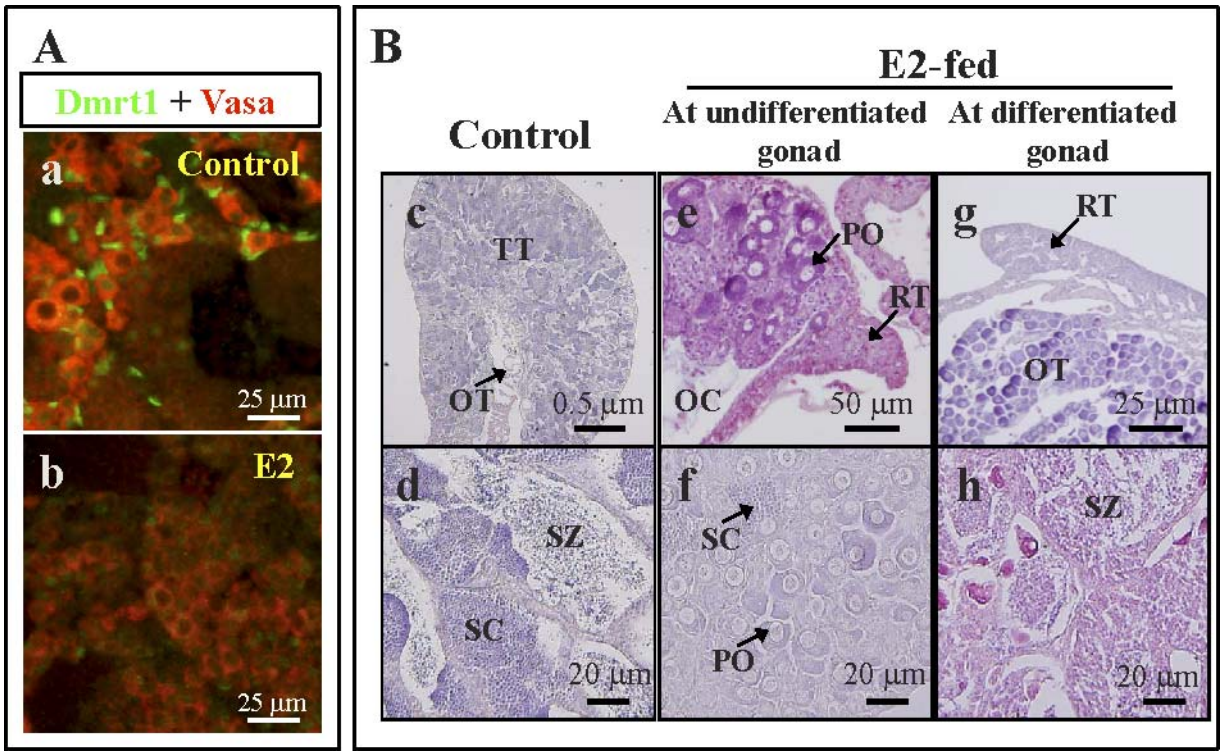

FIG. 4. Effects of E2 during gonadal development. Fish at stage 3 received E2 (2 μg/g body wt, two i.p. injections in 5 days) to examine the short-term effects of E2 on *dmrt1* expression. IF staining of Dmrt1 in control (**a**) and E2-injected fish (**b**) is shown (**A**). **B**) E2 (6 mg/kg feed) was given orally during different periods. In strategy I, E2 was given from June (stage 1; undifferentiated gonad) to September (stage 3; age, 3–6 mo), followed by E2 withdrawal from September to January of the next year. In strategy II, E2 was given from July (stage 2; differentiated gonad) to October (stage 3; age, 5–8 mo), followed by E2 withdrawal from October to January of the next year (age, 1 yr). Hematoxylin-and-eosin staining indicated the ovotestis from controls at the age of 1 yr (**c** and **d**), E2-treated fish in strategy I at the age of 6 mo (E2-treatment; **e**), 10 mo (E2-termination; **f**), E2-treated fish in strategy I at the age of 8 mo (E2-treatment; **g**); and strategy II at the age of 1 yr (E2-termination) (**h**). OT, ovarian tissue; PO, primary oocyte; RO, regressed oocyte; RT, regressed testicular tissue; SC, spermatocyte; SZ, spermatozoa; TT, testicular tissue.

and spermatocytes and not in Sertoli cells [30]. In zebrafish, *dmrt1* is expressed in the germ cells in both sexes [37]. Taken together, these results suggest that *dmrt1* not only regulates Sertoli cells but also plays a role in gametogenesis.

How does *dmrt1* mediate spermatogonial proliferation in the black porgy? A candidate gene that may regulate spermatogonial proliferation in the black porgy is *amh*. In the *dmrt1*-deficient black porgy, the expression of *amh* and its receptor, *amhr2*, was significantly decreased in the *dmrt1*-deficient testis compared to controls (data not shown). *Amh*-deficient male mice have testes that are fully descended and produce viable sperm [51]. Conversely, in medaka, loss of function of *amh* and *amhr2* results in the suppression of germ cell proliferation during gonadal differentiation in both sexes [52].

Furthermore, after busulfan treatment, we found that the suppression of germ cell proliferation without interference of *dmrt1* expression in Sertoli cells and other male-related genes (*amh* and *amhr2*) was the cause of the defects. In medaka, the germ cell-deficient gonad is masculinized and expresses *dmrt1* and *sox9b*; genotypic females do not express *amh* [53]. In medaka with the hotei mutation (*amhr2*-deficient), more than 50% of fish underwent sex reversal [54]. These results raise the possibility that *dmrt1* regulation is autonomous, without influence from factors released from the germ cells.

The Natural Sex Change Is Stimulated by the Loss of Male Function Through a Deficiency of dmrt1 *in Sertoli Cells*

When *dmrt1* expression is down-regulated in the testis, the germ cells are completely lost, and the fish undergoes a male-to-female sex change. We next asked whether the testis regulates ovarian development. Histological studies showed that the ovary develops during the postspawning period (March to May), when the testis regresses (with low *dmrt1* expression) (Fig. 3); the ovary then degenerates, while the testis regenerates (with high *dmrt1* expression; data not shown), during the nonspawning period (June to October) in males [4, 5]. Removal of the testis from the ovotestis induced ovary development, entry of the oocyte vitellogenesis, as well as a sex change in 1^{+}- to 2-yr-old fish [9–11]. In the present experiments, we further demonstrated that *dmrt1* deficiency (by exogenous E2 treatment and RNAi) results in testis regression and ovarian development with a sex change. Taken together, our present results and those of previous studies reveal that testis development is a key regulator of the male-to-female sex change in the ovotestis.

Male-Phase Maintenance Is Mediated by the Gnrh-Gth-Dmrt1 Axis

It is important of know which signals rescue *dmrt1* expression for testis development in males. We found that an LHRH analog induced *dmrt1* expression in vivo and that hCG increased *dmrt1* expression in vitro. Expression of *dmrt1* in the control was significantly decreased during the in vitro culture period, perhaps due to a lack of Gth stimulation. We also found an increase in serum Lh levels in 2^{+}- to 3-yr-old males as compared to sex-changing females. Increased serum Lh levels were also detected during the natural sex change from an ''E2-induced female'' to a male during E2 withdrawal in 1-yr-old

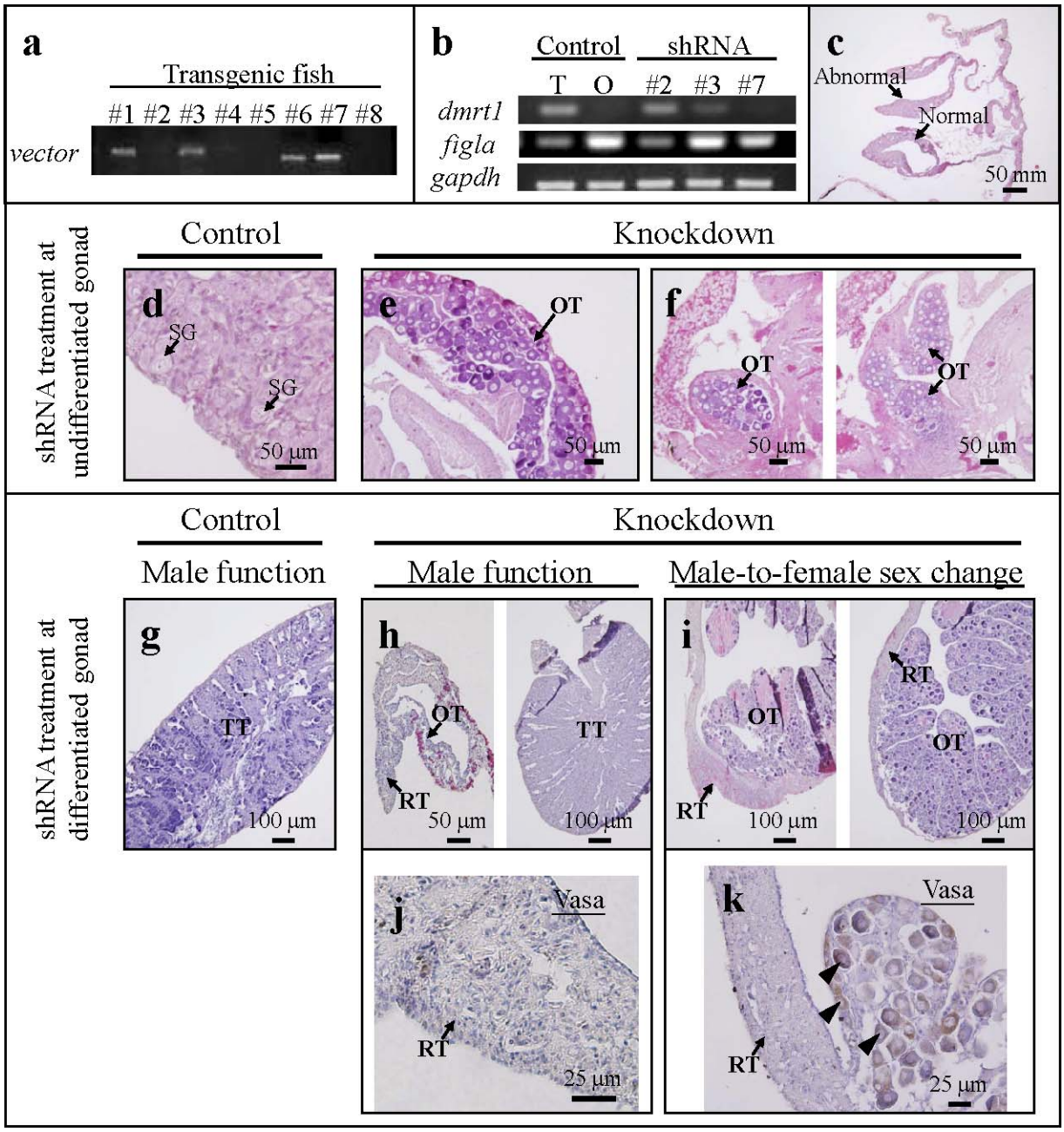

FIG. 5. Knockdown of *dmrt1* in the testis results in regression and germ cell loss. The gene-delivery ability was tested by PCR for the zebrafish U6 promoter (**a**). Levels of *dmrt1* and *figla* (female marker) were measured by Q-PCR in the gonadal tissue in controls and virus-injected fish (**b**). Sections of the pair gonads from a 6-mo-old transgenic fish carrying shRNA showed an abnormal gonad (no formation of ovarian cavity) and a normal gonad (**c**). After shRNA treatment in the undifferentiated gonad (stage 1), hematoxylin-and-eosin (H&E) staining demonstrated the sections of control gonads in 6-mo-old fish (stage 3; **d**) and suppressed testis with ovary growth in transgenic 6-mo-old fish (**e** and **f**). After shRNA treatment in the differentiated gonad (stage 2), H&E staining indicated the control gonads (**g**) and regressed testis (**h**) with ovary growth (**i**) in injected fish during the first spawning season (age, 11 mo). IHC was conducted for Vasa (brown) and counterstaining with hematoxylin (purple) in the gonads of injected fish with regressed testis (**j**) and ovary growth (**k**). Arrowhead indicates Vasa (for germ cells). OT, ovarian tissue; RT, regressed testis; SG, spermatogonia; TT, testicular tissue.

black porgy [8]. Exogenous Gth triggered the female-to-male sex change in the honeycomb grouper [55]. In mice, expression of *dmrt1* is strongly decreased in tissue culture, and Gth can rescue this loss in vitro [32]. These results suggest the signals that rescue *dmrt1* in the testis might be due to contribution of the brain-pituitary-gonadal axis. Therefore, we propose that the Gnrh-Gth-Dmrt1 axis may play a significant role in the control of sexual fate and favors the maintenance of the male phase. It is possible that the regression of testis (due to the lack of *dmrt1*) (Figs. 1–5) as well as surgical removal of the testis [9–11] both result in the relief of testicular inhibition and the facilitation of ovarian development in the ovotestis.

dmrt1 *as an Early Molecular Signal for the Natural Sex Change*

We also found in the present study that *dmrt1* plays an important role in the natural sex change (in 2- to 3-yr-old fish) and can act as an early marker of sexual fate, which indicates whether the fish will become a male or a female during the

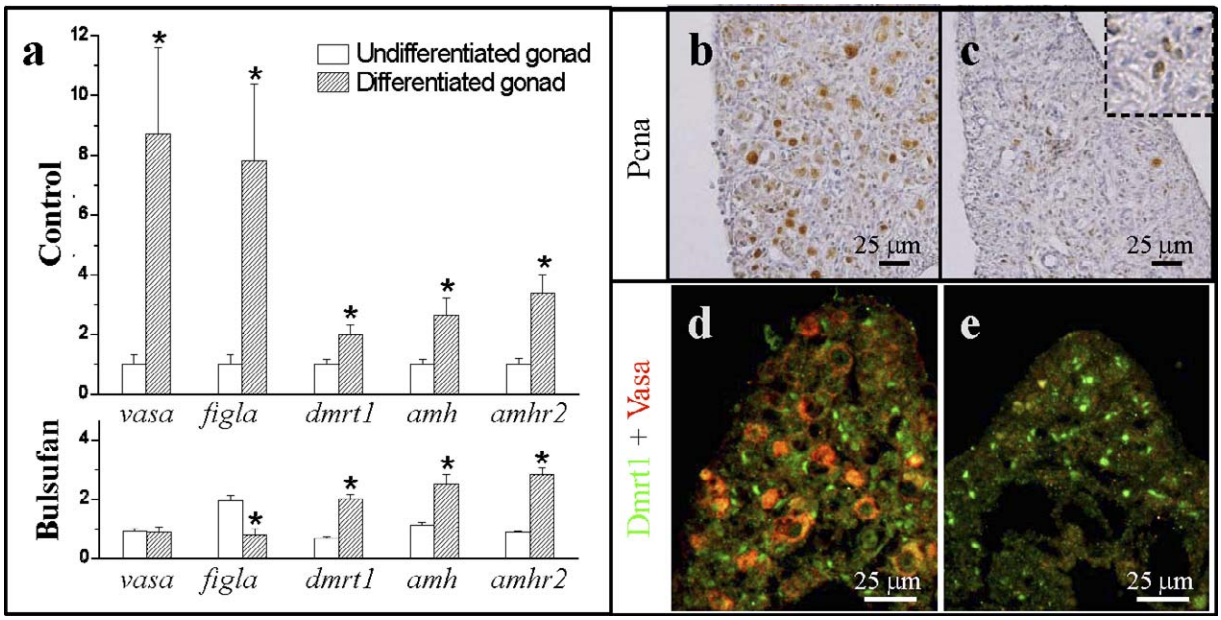

FIG. 6. Germ cells do not regulate *dmrt1* expression. Busulfan (20 mg/kg feed) was given to the fish from the age of 2 mo (stage 1) to 8 mo (stage 3). **a**) Real-time PCR results of gonadal marker genes in the control and busulfan-treated fish during gonadal differentiation. The marker genes were as follows: *dmrt1*, *amh*, and *amhr2* for male-related genes; *vasa* for germ cell marker; and *figla* for the female-related gene. The relative expression of the genes was calibrated with the internal control, *gapdh*. Asterisk indicates a Student *t*-test ($P < 0.05$). **b** and **c**) Cell proliferation of gonads was revealed by IHC for Pcna (brown) in the control (**b**) and busulfan-treated fish (**c**) in the differentiated testis (stage 2). **d** and **e**) IF staining for Dmrt1 (green) and Vasa (red) in the gonads of control (**d**) and busulfan-treated fish (**e**) in the differentiated gonad (stage 2).

third spawning period. This is dependent on the expression levels of *dmrt1* in the testicular tissue at the age of 2^+ yr (5–6 mo before the third spawning period). We propose that the fish will remain male if the testicular tissue has high levels of *dmrt1* expression but will change to a female (5–6 mo later) if the testicular tissue has low levels of *dmrt1* expression. Expression of *dmrt1* in the testicular tissue may predict whether the natural sex change will occur at the age of 2^+ to 3 yr.

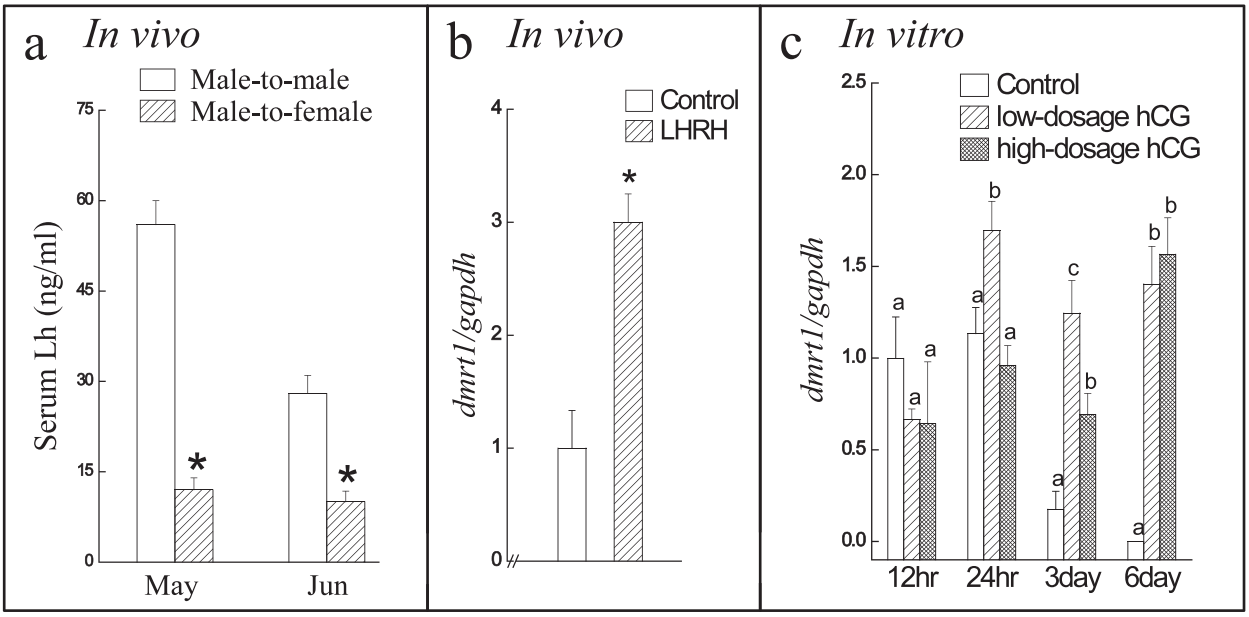

FIG. 7. Gonadotropin stimulates testicular *dmrt1* expression. **a**) Serum Lh concentrations in male-to-male fish and male-to-female sex change fish were measured during the post- and nonspawning season (stage 6; serum was collected 13 times from April to June). **b**) Fish were given an LHRH analog by intramuscular injection at stage 6 on Days 1 and 5 (two injections total). Gonads (testicular and ovarian tissues) were collected 24 h after the second injection (Day 6). Gonads were collected at stage 5, ovarian tissue was entirely removed from the ovotestis, and *dmrt1* expression was analyzed. Relative responses of the LHRH-injected fish were normalized to controls. **c**) The hCG (high dose, 2 IU/ml culture medium; low dose: 2×10^{-3} IU/ml culture medium) was administered for a 24-h period, and testicular tissue were collected at 12 h, 24 h, Day 3, and Day 6 for *dmrt1* analysis. The relative response of hCG-treated testicular tissue was normalized to control (12-h culture). Asterisks in **a** and **b** indicate a Student *t*-test ($P < 0.05$); superscript letters in **c** indicate one-way ANOVA and a Student-Newman-Keuls multiple test ($P < 0.05$).

In conclusion, the present study shows that Sertoli cell *dmrt1* is a key regulator for testis differentiation and development as well as for spermatogonial proliferation during testis development in the black porgy. These findings suggest that *dmrt1* is also important in testis growth/development and in the control of sexual fate. Finally, our results demonstrate that brain regulators (Gnrh and Gth) promote *dmrt1* expression and testis development to further prevent ovarian development in 2^{+}- to 3-yr-old fish. Our findings shed light on the mechanism of gonadal sex determination and sexual fate in the ovotestis that is controlled by the brain.

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