BOR Papers in Press. Published on November 28, 2012 as DOI:10.1095/biolreprod.112.104398

Title: Oocytes Survive in the Testis by Altering the Soma Fate from Male to Female in the Protandrous Black Porgy, *Acanthopagrus schlegeli*[1]

Running title: Oocyte Figla mediated the fate of surrounding cells

Guan-Chung Wu[2,4] and Ching-Fong Chang[3,4,5]

[4]Center of Excellence for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

[5]Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan

[1]Partially supported by the National Science Council and Ministry of Education (Taiwan).

[2]Correspondence: E-mail: gcwu@mail.ntou.edu.tw

[3]Correspondence: E-mail: B0044@mail.ntou.edu.tw

ABSTRACT

In fish, hermaphroditism is derived from gonochorism. No ancient ancestry and no single sex-determining mechanism are involved in the hermaphroditic fishes. Furthermore, hermaphroditic fish have a common set of transcriptional regulators that are involved in gonadal differentiation. However, the origins and evolution of hermaphroditism in fish remain far from understood. In the protandrous black porgy, the ovotestis is separated by connective tissue, and no intersex (ectopic located germ cells) characteristics are observed in either part. We generated the abnormal testicular part of the ovotestis with estradiol-17beta (E2) treatment, in which newly regenerated testis have ectopic oocytes. In this study, we performed a detailed phenotypic and molecular analysis of these E2-induced ectopic oocytes in the testicular part of the ovotestis. We showed that the oocytes in the regenerated testis do not undergo apoptosis, and thus, there are a number of oocytes in the testis. In these oocytes, Figla has a prolonged expression with ectopic expression of Cyp19a1a. Strikingly, the cells surrounding the oocytes are Dmrt1-positive cells (putative Sertoli cells) with high Figla expression in the oocytes at an early stage and then as the Dmrt1 expression diminishes, Cyp19a1a-positive cells (putative follicle cells) with low Figla expression appear in the oocytes at a later stage. This finding indicates that oocytes are competent to create a micro-environment to protect against a testicular environment in black porgy fish. Furthermore, Figla is likely the key factor in the pathway of Sertoli cell transformation into follicle-like cells. These results shed light on the reason why the presence of more than one sex at a time existed during an evolutionary transition from gonochorism to hermaphroditism in fish.

Summary sentence: Sertoli cells undergo transdifferentiation into follicle-like cells through the prolonging of Figla expression in the protandrous black porgy.

Keywords: transdifferentiation, Sertoli cells, ovotestis, sex change, Figla

INTRODUCTION

Fish are the only vertebrate class with functional hermaphroditism [1]. The capacity for sex change is lost from amphibians to mammals. Hermaphroditic fish are therefore polyphyletic and derive from gonochorism. Hermaphroditic fish have been documented in 2% of the 25,000 teleost species [2]. Furthermore, no hermaphroditic lineage appears to be evolutionally ancient [3]. However, most of our knowledge regarding sex change in fish is from studies of their endocrine characteristics. How the presence of more than one sex at a time existed during an evolutionary transition from gonochorism to hermaphroditism in fish remains unknown.

In mammals (mice), sex determination results from the initial switch of either the *Sry*-dependent testis-differentiating or *Sry*-independent ovary-differentiating molecular cascade in an exclusive manner [4]. However, the initiation of both pathways in the same gonad will result in the development of an ovotestis [5]. These ovotestes can be caused by an insufficient or delayed expression of the testis-related gene in the XY gonad [6-8] or ectopic expression of the testis-related gene in the XX gonad [9]. However, the ovarian region of the ovotestis undergoes apoptosis during late embryogenesis, thus leading to a normal testis at birth [9]. Loss of *Foxl2* in adult female mice results in reprogramming of the granulosa and theca cell lineages into Sertoli-like and Leydig-like cell lineages, even in normal oocytes [10]. Therefore, it has been shown that oocytes are not competent to create a micro-environment that protects against a testicular environment in mammals.

The black porgy (*Acanthopagrus schlegeli* Bleeker) is a protandrous hermaphroditic fish with a striking life cycle (Supplemental Fig. S1, available online at www.biolreprod.org). The fish has a stable pattern for sex change, where the first two reproductive cycles are 100% male, after which approximately 50% of individuals change sex in the third reproductive cycle to become female [11, 12]. Increased plasma estradiol-17β (E2) levels are correlated with the natural sex change in the third reproductive cycle [11], which can be blocked by long-term administration of aromatase inhibitor (AI) [13]. The sexual phase can be changed from male to female with long-term E2 treatment at any age; the ovotestis contains a degenerated testis and a developed ovary (dominant part) [11, 12, 14-21]. However, vitellogenic oocytes are infrequently observed following long-term E2 treatment [15, 21]. In addition, in <2-yr-old fish, a reversible sex change can occur in the E2-induced female, which can switch from an induced female to a male following the end of E2 administration (E2-termination) [12, 14-21]. In contrast, surgical elimination of the testis in the ovotestis results in the development of the ovarian part and the female phase (with vitellogenic oocytes) in 100% of >1-yr-old fish [12, 18, 19]. Therefore, our data reveal that the maintenance of the testis occurs through suppressed ovarian growth and the natural sex change [16]. However, an abnormal testis was observed in <1-yr-old fish that underwent a female-to-male sex reversal

after E2-termination [17]. The oocytes are broadly observed in the newly regenerated testicular part of the ovotestis after E2-termination in <1-yr-old fish [16]. Therefore, it may be possible for oocytes to create a micro-environment for protecting themselves against a testicular environment. Furthermore, Sertoli cells may undergo autonomous cell reprogramming into follicle-like cells upon facing the female micro-environment.

The oocyte itself regulates the development of ovarian follicles in mice [22]. Female germ cells do not survive when the oocytes fail to interact with the follicles cells, either due to their ectopic location [23] or in the absence of a critical oocyte-specific transcription factor, factor in the germline alpha (*Figla*) [24]. FIGLA is a basic helix-loop-helix transcription factor that plays a crucial role in the formation of primordial follicles. In *Figla*-mutated mice, embryonic gonadogenesis appears normal, but primordial follicles are not formed at birth, and massive depletion of oocytes results in female sterility [24]. Using a *Figla*-null mouse to analyze the downstream genes of *Figla* reveals that FIGLA functions as a key regulatory molecule in coordinating the expression of the NALP family of genes [25]. FIGLA also inhibits the expression of male germ cell-specific genes [25] and results in the impairment of meiosis and germ cell apoptosis [26]. These data implicate FIGLA as a central regulator of oocyte-specific genes that play important roles in folliculogenesis, fertilization, and early development. The expression of *figla* has been observed in early oogenesis in fish, including medaka [27], zebrafish [28], and black porgy [21]. Therefore, we hypothesized that *figla* is involved in the early ovarian development and is required for oocyte survival in a testicular environment in the protandrous black porgy.

We have previously generated the abnormal testicular part of the ovotestis by E2 treatment, in which newly regenerated testes contain ectopic oocytes [17]. In this study, we performed a detailed phenotypic and molecular analysis of this E2-induced ectopic location of the oocytes in the testicular part of the ovotestis. We showed that the oocytes in the regenerated testis did not undergo apoptosis and thus maintained a number of oocytes in the testis. In this regenerated testis, oocytes had a prolonged expression of Figla and ectopic expression of Cyp19a1a. Strikingly, the surrounding cells of the oocyte were Dmrt1-positive cells (putative Sertoli cells) with high Figla expression at an early stage then Cyp19a1a-positive cells (putative follicle cells) with low Figla expression appear at a later stage. This finding indicates that the oocytes are competent to create a micro-environment to protect against a testicular environment in the black porgy fish. Furthermore, Figla is the key factor in the process from Sertoli cells to follicle-like cells. These results provide the possible reason for the presence of more than one sex at a time during an evolutionary transition from gonochorism to hermaphroditism in fish.

MATERIALS AND METHODS

Experimental Fish

Juvenile black porgy fish were used in the experiments. The gonadal developmental stages are

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. The fish were fed with a commercial feed (Fwusow, Inc. Taichung, Taiwan) *ad libitum*. All procedures and investigations were approved by the National Taiwan Ocean University Institutional Animal Care and Use Committee and were performed in accordance with standard guiding principle.

Experimental Design

Experiment 1: *The characteristics of ectopically located oocytes in the testis.* In black porgy, testicular tissue and ovarian tissue are separated by connective tissue. We created an ectopic location of the oocytes in the testicular part of the ovotestis as described previously [17]. Fish were fed a diet containing E2 (6 mg/kg of feed) at the gonadal stage 1 (Supplemental Fig. S1; age, 2 mo) for 3 mo (short-term group, Fig. 1A) and for 9 mo (long-term group, Fig. 1A; age 11 mo). The short-term group was followed by the replacement of the E2 diet with control feed at the gonadal stage 2 (Supplemental Fig. S1; age, 5 mo) until the first spawning season. Samples (n = 8 fish/group) were collected monthly for genetic analysis and histology. Anti-Dmrt1 (Sertoli cells marker) and anti-Cyp19a1a (follicle cells marker) were used to identify the types of cells surrounding the oocytes.

Experiment 2: The fate of testicular somatic cells with reduced dmrt1 expression. We used a lentiviral approach to deliver shRNA designed to partly knock down *dmrt1* expression as described previously [17]. The virus was injected (i.p.) 3 times (1 week interval) during the gonadal stage 2 (differentiated ovotestis, Supplemental Fig. S1). Fish (n = 20) were collected three months after the last injection, and samples were collected for histology. Anti-Dmrt1 (a Sertoli cells marker) and anti-Cyp19a1a (a follicle cells marker) were used to identify the somatic cell types.

Experiment 3: Effects of sex steroids on figla expression. To determine the effects of sex steroids on *figla* expression, fish (n = 5 fish each in the treated and control groups) at gonadal stage 4 (Supplemental Fig. S1) were injected (i.p.) with E2 (1.5 μg/g of body weight) and methyltestosterone (MT, 1 μg/g of body weight) at 0, 2, and 4 days. Fish gonads (testicular and ovarian tissues) were collected 24 hr (day 5) after the third injection (day 4), and analysis of *figla* expressions was performed. Other fish were fed a diet containing E2 (6 mg/kg of feed) and AI (1,4,6-androstatriene-3,17-dione; 20 mg/kg of feed) at the gonadal stage 1 (Supplemental Fig. S1; age, 3 mo) until the size of the oocytes was greater than 30 μm. Fish gonads were collected for histology and analysis of *figla* expression.

Plasmid Construction and Virus Production

The vector for *dmrt1* knockdown was described in our previous study [17]. Virus production was performed according to the manufacturer's protocol (Clontech). The virus was kept at -80℃ until it was

used.

Antiserum Production

We used the synthesized peptide-ovalbumin conjugation to immunize the antiserum for Figla. The Figla antiserum was produced in white rabbits immunized against a peptide fragment of black porgy Figla (SNIEKFRRAKTGSYVC and CLMRPDRKPSKVDTLK). The antisera were prepared by Yao-Hong Biotechnology, Inc (New Taipei City, Taiwan). Extracts of the ovotestes were used to confirm the specificity of the antiserum by Western blot analysis as described by Wu and Chang [12, 17]. Immunoblotting was performed with preabsorbed antibodies at 4℃ overnight. Finally, the BCIP/NBT Liquid Substrate System (Sigma) was used to detect the protein staining. A specific and single protein band corresponding to black porgy Figla (according to the molecular weight) was detected.

In Situ Hybridization

We used antisense RNA probes for *figla*, and the *in situ* hybridization of tissue sections was performed as described previously [12].

Histological Analysis, IF Staining and IHC Staining

Hematoxylin-and-eosin staining and IHC and IF staining were performed as described previously [17, 21]. Fish gonads were fixed with 4% paraformaldehyde in PBS. Antisera of Figla (1:400), anti-Vasa (1:1000) [17], anti-Proliferating cell nuclear antigen (Pcna) (1:100, Santa Cruz Biotechnology), anti-Cyp19a1a (1:1500) [21], and anti-Doublesex and mab-3-related transcription factor 1 (Dmrt1; 1:3000) [17] were used for IHC and IF staining.

TUNEL Staining

Fish gonads were fixed with 4% paraformaldehyde in PBS. TUNEL staining was performed according to the manufacturer's protocol (Promega) as described previously [12]. DNaseI-treated slides were used as a positive control.

RNA Analysis

Gonads were collected and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). This homogenate was used for RNA analysis. Extraction of total RNA and synthesis of the first-strand cDNA were performed according to the manufacturer's protocol. Total RNA (1 μg) extracted from the gonad of a representative fish was reverse transcribed into first-strand cDNA using Superscript III (Invitrogen; Carlsbad, CA) with the oligo (dT) 12–18 primers. This first-strand cDNA was used for the quantitative real-time PCR (qPCR) analyses. Specific primers for *dmrt1* (GenBank accession no. AY323953), *figla* (GenBank accession no. EU496494), *cyp19a1a*, *amh* (*anti-Müllerian hormone*, GenBank accession no.

GU256046), and *cyp11b2* (*11β-hydroxylase*, GenBank accession no. EF423618) were described previously [12, 17-19, 21]. qPCR was performed as described previously [19]. All samples were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*, GenBank accession no. DQ399798), and maximum values for each group were defined as 100.

Data Analysis

All data are expressed as means ± SEM (standard error of mean). 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 among 3 treatments. Student *t*-test was also conducted to determine significant differences ($P < 0.05$) between 2 treatments.

RESULTS

Exogenous Estrogen Not Only Inhibited Testicular Development but Also Altered the Fate of the Germ Line in Testicular Tissue during Early Gonadal Development

Fig. 1A is the schematic picture to show the histological characters (Fig. 1B). In the control fish, ovotestes were observed in 3.5-mo-old fish. Lobular testicular tissue with spermatogonia appeared in 5-mo-old fish (Fig. 1Aa/Ba). Active spermatogenesis (with spermatocytes and spermatozoa) was observed in 8-mo-old fish (Fig. 1Ab/Bb) and in the fish entering the male phase in the first spawning season (11-mo-old fish) (Fig. 1Ac/Bc). Ovarian tissue was formed in a small portion of the ovotestis in the first reproductive cycle (Fig. 1Aa-c/Ba-c). Among fish that were treated with long-term E2 administration (LT-group), ovarian tissue with many primary oocytes was observed together with regressed testicular tissue in 5-mo-old fish (Fig. 1Ad/Bd). After 6 mo of E2 administration (8-mo-old fish), ovarian tissue had formed in a large portion of the ovotestis with completely regressed testicular tissue (Fig. 1Ae/Be). Oocytes did not enter the secondary oocyte stage or vitellogenesis in the first spawning season (Fig. 1Af/Bf). Among fish treated with short-term E2 administration (ST-group, estrogen treatment was terminated in 5-mo-old fish after 3 mo of E2 feeding), E2 administration induced ovarian development (similar to Fig. 1Ad/Bd), and newly generated testicular tissue was observed after 3 mo of E2 termination (8-mo-old fish) (Fig. 1Ag/Bg). The testicular tissue showed oocytes (ectopic location) together with male germ cells (Fig. 1Bg"). Oocyte diameters did not differ between normal oocytes (60.9 ± 0.72 μm) and those that were expressed ectopically (58.1 ± 1.15 μm). After 6 mo of E2 termination (11-mo-old fish), fish reversed sex from female to male and entered the male phase (Fig. 1Ah/Bh).

Ectopic Expression of Female-Related Genes in the Testis Is Associated with Abnormal Location of Oocytes

In the ST-group (short-term treatment, with 3 mo of E2 treatment and 3 mo of E2-termination) in 8-mo-old fish, we observed ectopic oocytes in the testis (Fig. 1g). Furthermore, we precisely separated the

testicular and ovarian parts of the ovotestis for gene expression. qPCR confirmed that male-related gene (*dmrt1*, *amh*, and *cyp11b2*) expression in the E2-terminated testis was not different compared to control fish with a normal testis (Fig. 2). In contrast, female-related gene (*figla* and *cyp19a1a*) expression was significantly increased compared to control fish (Fig. 2). Additionally, *figla* and *cyp19a1a* expression in the E2-terminated testis was lower than in the E2-terminated ovary.

Figla Is Localized in Early Primary Oocytes of Limited Size

Transcripts of *figla* showed the robust expression at the early primary oocyte stage, and *figla* expression in the oocytes was decreased at a later stage (Fig. 3a). We next examined the localization of Figla by IHC staining using a specific anti-Figla antibody. The specificity of the antibody to Figla was confirmed by a Western blot analysis using ovotestis extracts (Fig. 3b). Figla expression was only observed in the ovary and not in the testis (Fig. 3b). IHC staining confirmed that Figla was robustly expressed in early primary oocytes but not in oogonia, and the expression was decreased in the late stage of the oocytes (Fig. 3c-e). Furthermore, qPCR confirmed that *figla* expression in gonocytes (undifferentiated gonad, 3-mo-old fish) and oogonia (differentiated ovotestis, 4-mo-old fish) was low and significantly increased in early primary oocytes (developed ovary, 5-mo-old fish) (Fig. 3f) in the short-term E2-fed fish (Experiment 1). According to our qPCR, ISH and IHC results, Figla was expressed in oocytes of limited size. The robust expression of Figla was observed in small oocytes and then decreased when the oocyte became larger than 28.6 μm (Fig. 3g).

Ectopic Female Germ Cell is Active during Testis Regeneration and Strongly Expresses Figla

According to IHC staining of Pcna (Fig. 4a and 4c) and Figla (Fig. 4b and 4d) in the serial sections, our results revealed that the female germ cells can highly proliferate in the regenerated testis of 7-mo-old fish (after 2 mo of E2 termination). Furthermore, the TUNEL assay revealed that ectopic oocytes and their surrounding cells did not undergo programmed cell death (apoptosis) in a testicular environment (Fig. 4e). The serial sections of Fig. 4e were used to confirm the activity of the TUNEL assay by DNase I treatment and the distribution of cells (Fig. 4f). Furthermore, the oocyte number in the regenerated testis was not significantly different between fish 3 mo (8-mo-old fish) and 6 mo (11-mo-old fish) after E2 termination (1138 ± 150 and 1366 ± 364 oocytes, respectively). Interestingly, Figla had a prolonged expression in oocytes which were ectopically expressed compared with normal oocytes in the ovary (Fig. 4g). The size of the Figla-expressing oocytes was calculated using imaging software; ectopic oocytes (< 48.4 μm) in the testis had a prolonged expression of Figla compared with normal oocytes (< 28.6 μm) in the ovary (Fig. 4g).

Ectopic Expression of Cyp19a1a in the oocyte cytoplasm and Sertoli-like Surrounding Cells in Ectopic Oocytes

In the normal ovary, follicle cell expression was Cyp19a1a-positive (Fig. 5a) and Dmrt1-negative (Fig. 5b). According to IHC staining of Cyp19a1a (Fig. 5c-e) in 8-mo-old fish (after 3 mo of E2 termination), our results revealed that small oocytes (33.9 ± 0.71 μm) have high expression levels of Cyp19a1a in the oocyte cytoplasm (Fig. 5c-e) compared with larger oocytes (43.3 ± 0.7 μm) and had high expression levels of Cyp19a1a in the follicle cells (Fig. 5a). Furthermore, ectopic oocytes with the expression of Cyp19a1a were only observed in the regenerating testis but not in the normal ovary (Fig. 5a and 5c-e). However, these ectopic oocytes were surrounded by Dmrt1-expressing cells (Sertoli cells) in the testis of E2-terminated fish (Fig. 5f and 5g). Furthermore, IF staining of Dmrt1 confirmed that ectopic oocytes are surrounded by Dmrt1-expressing cells in the testis (Fig. 5h and 5i). Taken together, these findings further support a model that those ectopic oocytes can antagonize the male environment.

Appearance from Sertoli Cells to Follicle-like Cells Related to Prolonged Expression of figla

In the ST-E2 administration group, our results showed that ectopic oocytes are surrounded by Cyp19a1a-expressing and Dmrt1-expressing cells (Fig. 5). To determine whether these two genes are expressed in the same cells or in different cells, IHC staining of Figla, Cyp19a1a, and Dmrt1 were performed in serial sections. The expression of Figla, Cyp19a1a, and Dmrt1 had a stable pattern. Figla was more highly expressed in oocytes in the presence of surrounding cells with Dmrt1-positive and Cyp19a1a-negative expression (Fig. 6a-c). In contrast, compared to oocytes with high Figla expression, oocytes with low Figla expression were surrounded by cells that had Dmrt1-negative and Cyp19a1a-positive expression (Fig. 6d-f). Furthermore, we used imaging software to determine the correlation between Figla, Cyp19a1a, and Dmrt1. We showed that the transcripts of Cyp19a1a and Dmrt1 in the surrounding cells have a stable expression during oocyte development (Fig. 6g). Dmrt1 was expressed in the early primary oocyte stage (< 32.4 ± 1.03 μm) and was significantly decreased in the later stage (> 43.5 ± 1.55 μm) (Fig. 6g). In contrast, Cyp19a1a expression was suppressed in the early primary oocyte stage (< 33.9 ± 0.71 μm) and was significantly increased in the later stage (> 43.3 ± 0.7 μm) (Fig. 6g).

Expression of figla *in the Ovary is Independent of Sex Steroids*

The expression of *figla* transcripts in the ovary did not differ after short-term E2 and MT treatment compared to the respective controls (Fig. 7a). Furthermore, results from IHC staining of Figla revealed that the female germ cells in the sex steroid-treated fish had a similar expression pattern to control fish. Figla was robustly expressed at the limited oocyte stage, and its expression decreased at the late oocyte stage (>28.6 μm) (data not shown). Additionally, AI- and E2-induced ovaries had a similar expression level of *figla* after 5 mo of treatment (Fig. 7b). At this period, the diameters of the oocytes in the treated fish were over 30 μm.

Knockdown of dmrt1 *can Lead to Germ Cell Depletion but not to Feminization in the Testis*

Control fish had a normal ovotestis that contained a large portion of mature testis and a tiny portion of regressed ovary that were separated by connective tissue (stage 3). All control fish had a normal male-phase gonad. Most transgenic fish also had a normal ovotestis. However, 3 of 20 transgenic fish had an abnormal gonad with a significantly reduced number of germ cells. The type of somatic cells in the *dmrt1*-deficient testis was examined; Dmrt1-expressing cells were only observed in control fish (Fig. 8a) but were not observed in knockdown fish (Fig. 8b). Cyp19a1a-expressing cells in the degenerated testis of the transgenic fish had similar characteristics to interstitial cells in the normal testis of the control fish (Fig. 8c and 8d).

DISCUSSION

Hermaphroditic fish have a common set of transcriptional regulators that are involved in gonadal differentiation. However, the origins and evolution of hermaphroditism in fish are far from understood. In hermaphroditic black porgy, the ovotestis is separated by connective tissue, and no intersex (ectopically located germ cells) is observed in either part. The present study was undertaken to systemically characterize the expression and function of the *figla* gene in an animal model. We demonstrated that oocytes are competent to create a micro-environment to protect against a testicular environment in black porgy fish. Furthermore, Figla is the key factor in the pathway of the appearance from Sertoli cells to follicle-like cells. These results potentially provide the reason for the presence of more than one sex at a time during an evolutionary transition from gonochorism to hermaphroditism in fish.

The Germ Lines of Both Sexes can be Developed Synchronously in the Regenerated Testis During Reversed Female-to-male Sex Change

In mammals, the follicular cells in oocyte-depleted follicles survive, proliferate, and subsequently acquire the morphological and genetic characteristics of Sertoli cells [29]. Germ cell-deficient fish usually develop as phenotypic males (medaka [30]; zebrafish [31]). Thus, the presence of germ cells is generally considered to be essential for female gonadal differentiation and for the maintenance of ovarian structure. In contrast, loach fish (*Misgurnus anguillicaudatus*), which lack germ cells, could develop as either phenotypic males or females with testicular or ovarian structures, respectively [32]. When the germ lines were sparse through the busulfan treatment, the gonad has normal testicular somatic gene expression and ovarian structure (ovarian cavity), indicating that the germ line does not influence fate determination of the surrounding somatic tissues in black porgy [17]. Thus, the soma and germ lines might have different regulating mechanism among teleosts.

Both E2 stimulation (E2 feeding) and E2 depletion (AI feeding) can induce male-to-female sex change in <1-yr-old black porgy fish [21]. Similarly, both treatments result in the suppression of the testis and the

development of a dominant ovary [21]. However, broad distributions of oocytes were only observed in the regenerated testis of the E2-terminated group [17]. In gonochoristic fishes, ectopic oocytes were only observed in the testis during long-term estrogen exposure [33, 34]. Furthermore, our previous studies showed that a proliferating marker (Pcna) was not expressed in the suppressed testis during E2 administration [17]. Both markers (Pcna and Figla) were observed in the regenerated testis after E2 termination. Thus, we speculate that E2 can disarrange the fate of germ cells in the testis. In mice, ectopic germ cells in the mesonephrous all differentiate into oocytes as they normally would in the ovary, even in males [35]. This result reveals that germ lines can spontaneously differentiate into oocytes without suitable environments in black porgy. However, proliferating germ cells may suppress a male pathway and activate a female pathway in the surrounding somatic cells, as shown in black porgy and hotei medaka fish [36]. Furthermore, the expression of *dmrt1* (a testicular differentiation regulatory factor) was suppressed by E2 treatment in the black porgy [17]. Thus, we suggest that E2 function is the dysfunction of Sertoli cells in the testis and that part of the germ cells spontaneously differentiate into oocytes.

Oocyte Expression of Figla Is Required for Primordial Follicle Formation

Our previous studies showed that figla expression is correlated with ovarian differentiation [21]. In the oocyte, the expression of *figla* is observed to significantly increase during early oocyte development [21]. Our present IHC staining results also revealed that Figla was robustly expressed in early primary oocytes. Compared with early primary oocytes, Figla was weakly expressed in early oogonia (<7.14 μm) and late primary oocytes (>28.6 μm) in black porgy. *FIGLA* genes are expressed in the fetal ovary, and that expression is significantly increased by the time of primordial follicle formation in mammals [37, 38]. In *Figla*-mutated mice, embryonic gonadogenesis appeared normal, primordial follicles were not formed at birth, and massive depletion of oocytes resulted in female sterility [24]. In fish, the expression of *figla* has been observed in early ovary development, including in medaka [27], zebrafish [28], and black porgy [21]. Taken together, these data implicate FIGLA as a central regulator of oocyte-specific genes that plays important roles in folliculogenesis and early development through evolution.

Transdifferentiation of Sertoli Cells Is Dependent on Oocytes Regulation Through Figla Expression

The role of DMRT1 in testis development is conserved throughout vertebrates [39]. In mice, loss of *Dmrt1* in the adult testis activates *Foxl2* and causes reciprocal sex transformation from Sertoli cells to granulosa cells [40]. *Cyp19a1a* is strongly expressed in the *Dmrt1*-mutant gonad and leads to germ cell feminization [40]. In black porgy, the loss of *dmrt1* in the testis can disrupt male development and is associated with sex change [17]. These results indicate that *Dmrt1* is essential for postnatal sex maintenance. Furthermore, *dmrt1*-deficient black porgy have normal Cyp19a1a expression (in interstitial cells) but never show oocytes in the testis. These results reveal that the loss of *dmrt1* cannot lead to the appearance of follicle-like cells and germ cell feminization in black porgy.

Our present studies showed that the cells surrounding ectopic oocytes begin as Sertoli (Dmrt1-positive) cells and then follicle-like (Cyp19a1a-positive) cells appear. In mice, follicle cells in oocyte-depleted follicles survive, proliferate, and subsequently acquire the morphological characteristics of Sertoli cells [29]. In the wrasse fish, some functional somatic cells of the ovary are reused as testicular somatic cells during the gonadal sex change [41]. In the medaka fish, the maintenance of *cyp19a1*-expressing cells was depended on oocytes [42]. These results suggest that the absence of oocytes caused the follicle cells to directly transdifferentiate into Sertoli-like cells. How do ectopic oocytes mediate Sertoli cells transdifferentiation to follicle-like cells in the black porgy? In mammals, a number of germ cell-specific expressed transcriptional regulators (*Figla*, *Nobox*, *Sohlh1* and *Sohlh2*) are critical to ovarian formation and folliculogenesis [43]. One of these transcriptional regulators, Figla, plays a crucial role in regulating early follicle formation in the black porgy. In the present study, ectopically located oocytes extended Figla expression longer in larger oocytes in the testis compared with normal oocytes in the ovary. Figla expression in ectopic oocytes was higher when the oocytes were surrounded by Sertoli cells than when they were surrounded by follicle-like cells. Moreover, the TUNEL assay revealed that Sertoli cells did not enter apoptosis. These results suggest that Figla by itself is sufficient to induce Sertoli cells to undergo transdifferentiation into follicle-like cells. In mammals, oocytes are directly required to maintain somatic cell identity during ovarian development [44, 45]. Female germ cells fail to interact with the follicle cells due to the ectopic location [23]. The ovarian region of the ovotestis undergoes apoptosis during late embryogenesis, thus leading to a complete testis at birth [9]. Taken together, these suggest that the mechanism for reprogramming the surrounding cells of the oocytes is mechanically varied among species. We found that Figla plays important roles in the transdifferentiation of the cells surrounding the germ cells in the black porgy.

Prolonging Expression of Figla in the Incorrect Micro-environment

It is important to know which signals extend Figla expression for oocyte maintenance in the incorrect environment. In contrast to eutherian mammals, estrogen is known to be important for ovarian differentiation and is able to trigger male-to-female sex reversal in non-eutherian vertebrates [46]. However, we found that high levels of androgen and estrogen and low levels of estrogen could not influence *figla* expression *in vivo* in the present studies. Similar to the control group, the expression of Figla in the ovary appeared in the early primary oocytes and did not extend the expression at the later stage during sex steroid treatment. These results reveal that *figla* expression is not regulated by exogenous sex steroids. However, high Figla expression was found in the surrounding cells (Sertoli-like cells) of the ectopic oocytes. On the basis of indirect evidence, it is proposed that *figla* is triggered by the incorrect surrounding of Sertoli cells. In medaka fish, *amh* expression is not detected in the somatic cells of the germ cell-deficient gonad [30]. Furthermore, in the condition of lacking germ cells, none of the XY *hotei*

mutants (*amhr2* mutant) exhibited female sex reversal [47]; on the contrary, XY *hotei amhr2* mutants but with germ cells had female sex reversal [47]. Taken together, these data indicate that in addition to a unidirectional signal, there is reciprocal cross talk between germ cells and somatic cells that plays an important role in sexual differentiation.

In conclusion, the present studies demonstrated that the surrounding cells (Sertoli-like) of ectopically located oocytes undergo transdifferentiation into follicle-like cells with the prolonged expression of *figla* in the testis. These findings are documented for the first time in the *in vivo* lineage reprogramming in an adult organism. Our data support the idea that prolonged expression of *figla* may help oocytes to antagonize the male environment and favor reprogramming of the fate of the surrounding cells. This characteristic might be the mechanism of an evolutionary transition from gonochorism to hermaphroditism in fish.

ACKNOWLEDGMENT
The manuscript was edited by the American Journal Experts.

REFERENCES

1. Policansky D. Sex change in plants and animals. Annu Rev Ecol Syst 1982; 13: 471-495.
2. Pauly D. Darwin's Fishes. Cambridge university press, cambridge 2004.
3. Avise JC, Mank JE. Evolutionary perspectives on hermaphroditism in fishes. Sex Dev 2009; 3: 152-163.
4. Kim Y, Capel B. Balancing the bipotential gonad between alternative organ fates: a new perspective on an old problem. Dev Dyn 2006; 235: 2292-2300.
5. Eicher EM, Washburn LL. Does one gene determine whether a C57BL/6J-Y(POS) mouse will develop as a female or as an hermaphrodite? J Exp Zool 2001; 290: 322-326.
6. Bagheri-Fam S, Sim H, Bernard P, Jayakody I, Taketo MM, Scherer G, Harley VR. Loss of Fgfr2 leads to partial XY sex reversal. Dev Biol 2008; 314: 71-83.
7. Bullejos M, Koopman P. Delayed Sry and Sox9 expression in developing mouse gonads underlies B6-Y(DOM) sex reversal. Dev Biol 2005; 278: 473-481.
8. Kim Y, Bingham N, Sekido R, Parker KL, Lovell-Badge R, Capel B. Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. Proc Natl Acad Sci U S A 2007; 104: 16558-16563.
9. Gregoire EP, Lavery R, Chassot AA, Akiyama H, Treier M, Behringer RR, Chaboissier MC. Transient development of ovotestes in XX Sox9 transgenic mice. Dev Biol 2011; 349: 65-77.
10. Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier AC, Klugmann C, Klasen C, Holter NI, Riethmacher D, Schütz G, Cooney AJ, Lovell-Badge R, Treier M. Somatic sex

reprogramming of adult ovaries to testes by FOXL2 ablation. Cell 2009; 139: 1130-1142.
11. 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.
12. 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.
13. 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.
14. Lau EL, Lin BY, Lee FY, Sun LT, Dufour S, Chang CF. Stimulation of testicular function by exogenous testosterone in male protandrous black porgy, *Acanthopagrus schlegeli.* J Fish Biol 1997; 51: 327-333.
15. 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.
16. Wu GC, Chang CF. The switch of secondary sex determination in protandrous black porgy, *Acanthopagrus schlegeli*. Fish Physiol Biochem 2012: DOI: 10.1007/s10695-10012-19618-10690.
17. Wu GC, Chiu PC, Lin CJ, Lyu YS, Lan DS, Chang CF. Testicular dmrt1 is involved in the sexual fate of the ovotestis in the protandrous black porgy. Biol Reprod 2012; 86: 41.
18. Wu GC, Chiu PC, Lyu YS, Chang CF. The expression of amh and amhr2 is associated with the development of gonadal tissue and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol Reprod 2010; 83: 443-453.
19. 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.
20. Wu GC, Tomy S, Lee MF, Lee YH, Yueh WS, Lin CJ, Lau EL, Chang CF. Sex differentiation and sex change in the protandrous black porgy, *Acanthopagrus schlegeli.* Gen Comp Endocrinol 2010; 167: 417-421.
21. 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.
22. Eppig JJ, Wigglesworth K, Pendola FL. The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc Natl Acad Sci U S A 2002; 99: 2890-2894.
23. Zamboni L, Upadhyay S. Germ cell differentiation in mouse adrenal glands. J Exp Zool 1983; 228: 173-193.
24. Soyal SM, Amleh A, J. D. FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. Development 2000; 127: 4645-4654.
25. Joshi S, Davies H, Sims LP, Levy SE, Dean J. Ovarian gene expression in the absence of FIGLA, an

oocyte-specific transcription factor. BMC Dev Biol 2007; 7:67.
26. Hu W, Gauthier L, Baibakov B, Jimenez-Movilla M, Dean J. FIGLA, a basic helix-loop-helix transcription factor, balances sexually dimorphic gene expression in postnatal oocytes. Mol Cell Biol 2010; 30: 3661-3671.
27. Kanamori A, Toyama K, Kitagawa S, Kamehara A, Higuchi T, Kamachi Y, Kinoshita M, Hori H. Comparative genomics approach to the expression of figalpha, one of the earliest marker genes of oocyte differentiation in medaka (*Oryzias latipes*). Gene 2008; 423: 180-187.
28. Jørgensen A, Morthorst JE, Andersen O, Rasmussen LJ, Bjerregaard P. Expression profiles for six zebrafish genes during gonadal sex differentiation. Reprod Biol Endocrinol 2008; 6: 25.
29. Guigon CJ, Coudouel N, Mazaud-Guittot S, Forest MG, Magre S. Follicular cells acquire sertoli cell characteristics after oocyte loss. Endocrinology 2005; 146: 2992-3004.
30. Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, Morohashi K, Tanaka M. Germ cells are essential for sexual dimorphism in the medaka gonad. Proc Natl Acad Sci U S A 2007; 104: 16958-16963.
31. Siegfried KR, Nüsslein-Volhard C. Germ line control of female sex determination in zebrafish. Dev Biol 2008; 324: 277-287.
32. Fujimoto T, Nishimura T, Goto-Kazeto R, Kawakami Y, Yamaha E, Arai K. Sexual dimorphism of gonadal structure and gene expression in germ cell-deficient loach, a teleost fish. Proc Natl Acad Sci U S A 2010; 107: 17211-17216.
33. Kipfer S, Segner H, Wenger M, Wahli T, Bernet D. Long-term estrogen exposure of whitefish *Coregonus lavaretus* induces intersex but not Lake Thun-typical gonad malformations. Dis Aquat Org 2009; 84: 43-56.
34. Urushitani H, Katsu Y, Kato Y, Tooi O, Santo N, Kawashima Y, Ohta Y, Kisaka Y, Lange A, Tyler CR, Johnson RD, Iguchi T. Medaka (*Oryzias latipes*) for use in evaluating developmental effects of endocrine active chemicals with special reference to gonadal intersex (testis-ova). Environ Sci 2007; 14: 211-233.
35. Upadhyay S, Zamboni L. Ectopic germ cells: natural model for the study of germ cell sexual differentiation. Proc Natl Acad Sci U S A 1982; 79: 6584-6588.
36. 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.
37. Bayne RA, Martins dSSJ, Anderson RA. Increased expression of the FIGLA transcription factor is associated with primordial follicle formation in the human fetal ovary. Mol Hum Reprod 2004; 10: 373-381.
38. Liang L, Soyal SM, Dean J. FIGalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. Development 1997; 124: 4939-4947.

39. Herpin A, Schartl M. Dmrt1 genes at the crossroads: a widespread and central class of sexual development factors in fish. FEBS J 2011; 278: 1010-1019.
40. Matson CK, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D. DMRT1 prevents female reprogramming in the postnatal mammalian testis. Nature 2011; 476: 101-104.
41. Nozu R, Horiguchi R, Murata R, Kobayashi Y, Nakamura M. Survival of ovarian somatic cells during sex change in the protogynous wrasse, Halichoeres trimaculatus. Fish Physiol Biochem 2012; (in press), published online ahead of print 16 March 2012 DOI: 10.1007/s10695-012-9632-2.
42. Nakamura S, Kurokawa H, Asakawa S, Shimizu N, Tanaka M. Two distinct types of theca cells in the medaka gonad: germ cell-dependent maintenance of cyp19a1-expressing theca cells. Dev Dyn 2009; 238: 2652-2657.
43. Jagarlamudi K, Rajkovic A. Oogenesis: transcriptional regulators and mouse models. Mol Cell Endocrinol 2012; 356: 31-39.
44. Guigon CJ, Magre S. Contribution of germ cells to the differentiation and maturation of the ovary: insights from models of germ cell depletion. Biol Reprod 2006; 74: 450-458.
45. McLaren A. Development of the mammalian gonad: the fate of the supporting cell lineage. Bioessays 1991; 13: 151-156.
46. Guiguen Y, Fostier A, Piferrer F, Chang CF. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol 2010; 165: 352-366.
47. Nakamura S, Watakabe I, Nishimura T, Picard JY, Toyoda A, Taniguchi Y, di Clemente N, Tanaka M. Hyperproliferation of mitotically active germ cells due to defective anti-Müllerian hormone signaling mediates sex reversal in medaka. Development (Camb) 2012; 139: 2283-2287.

FIGURE LEGENDS

Fig. 1. E2 effects in relation to gonadal development in the black porgy. **A**) Annual profiles of gonadal tissue in fish undergoing E2 administration and E2 termination from the juvenile to the first spawning season. Testicular tissue [TT] in black; ovarian tissue [OT] in gray; regressed testicular tissue [RT]. The gonadal stages were defined as follows: **a**) active testis with spermatogonia and inactive ovary with the primary oocytes; **b**) active testis with spermatogenesis and inactive ovary with the primary oocytes; **c**) mature testis; **d**) inactive testis and active ovary with the early primary oocytes; **e** and **f**) inactive testis and active ovary with the primary oocytes; **g**) regenerated testis with spermatogenesis and ectopic oocytes and inactive ovary with the primary oocytes; **h**) mature testis and regressed ovary with the primary oocytes. Fish were fed a diet containing E2 for 3 mo (short-term group) or 9 mo (long-term group). **B**) Histology of the gonad at different developmental stages as shown in **A**. **g"**) ectopic oocytes in the regenerated testis. An arrowhead indicates ectopic oocytes. LT group, long-term E2 administration group; ST group, short-term E2 administration group; OC, ovarian cavity.

Fig. 2. The expression profiles of sex-related genes in the E2-terminated regenerated testis. In the control group, fish had an active testis with spermatogenesis (testicular tissue, TT). In the E2-termination group, fish had a regenerated testis with the spermatogenesis and ectopic oocytes. The ovary was maintained in an inactive stage with primary oocytes. Testicular tissue with ectopic oocytes (E2-termination, TT) and ovarian tissue were separated to analyze the gene expression levels. The transcripts of sex-related genes (male-related genes: *dmrt1*, *amh*, and *cyp11b2*; female-related genes: *figla* and *cyp19a1a*) were calibrated with the internal control, *gapdh*, and then normalized (the value in the highest one of each gene was 100%). Lowercase letters indicate a one-way ANOVA and a student-Newman-Keuls multiple test ($P < 0.05$). Asterisks indicate a Student *t*-test ($P < 0.05$).

Fig. 3. The expression of *figla*/Figla in the oocytes during ovarian development. Localization and expression of *figla*/Figla are shown. **a**) *in situ* hybridization analysis of *figla* in the ovary. **b**) Western blot analysis using a Figla antibody. Extracts from the testis (TT) and the ovary (OT) were examined by immunoblotting with a Figla antibody followed by an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G. Figla was only detected in the ovary. **c**-**e**) IHC analysis of the ovary with the Figla antibody. Figla was not expressed in the oogonia and was robustly expressed in the early primary oocyte and faintly expressed in the later oocyte stage. **f**) qPCR results show that *figla* expression was maintained at a low level of expression in the gonocytes (undifferentiated gonad, 3-mo-old fish) and oogonia (differentiated ovotestis, 4-mo-old fish) and significantly increased in the early primary oocytes (developed ovary, 5-mo-old fish) in the short-term E2-fed fish (Experiment 1). **g**) The profile of Figla expression in various stages of the oocytes. Figla was robustly expressed in the early primary oocytes. TT, testicular tissue; OT, ovarian tissue; PO, primary oocyte; OG, oogonia.

Fig. 4. Female germ cell proliferation and Figla expression pattern in the regenerated testis. **a**) IHC analysis of proliferating marker (Pcna) in the regenerated testis. **b**) The serial section of **a** and staining with Figla. **c** and **d**) High magnification of **a** and **b** to show that Pcna and Figla were co-expressed in the same cell (arrowhead). **e**) Apoptosis analysis revealed slight signals in the regenerated testis compared with the DNaseI treatment in the serial section with a robust and broad signal (**f**). **g**) In IHC analysis of the oocytes, Figla had a different expression pattern in the testicular part and ovarian part. Ectopic oocytes had the robust expression in the later oocyte stage of the regenerated testis compared to weak expression in the oocytes of the ovarian tissue. An arrowhead indicates Figla expression. TT, testicular tissue; OT, ovarian tissue.

Fig. 5. There were two types of cells surrounding the oocytes in regenerated testis. IHC analysis of positive Cyp19a1a (**a**) and Dmrt1 (**b**) in the ovarian tissue. **c**) IHC analysis of Cyp19a1a in the regenerated testis. **d** and **e**) High magnitude of **c**. Positive Cyp19a1a in the primary oocytes, (**f**) IHC

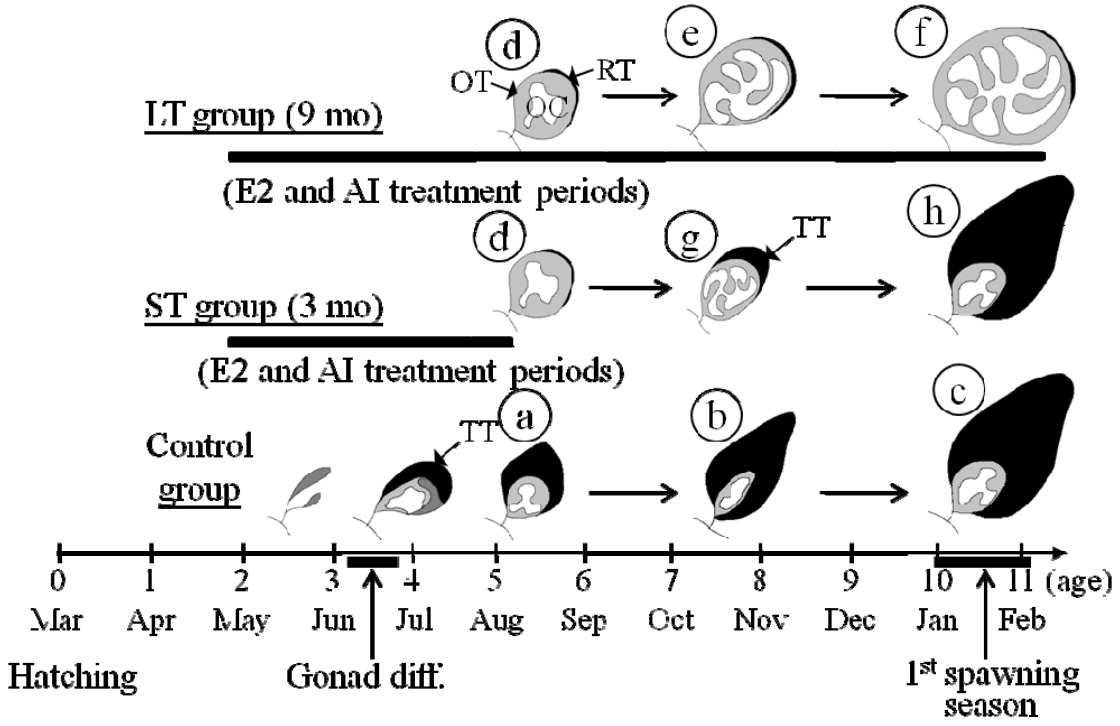
analysis of Dmrt1 in the regenerated testis. **g**) A high magnitude of **f** shows that the cells surrounding the oocyte were Dmrt1-positive. **h** and **i**) IF analysis of Dmrt1 and counterstained with DAPI showed that the oocytes were surrounded by Dmrt1-positive cells. An arrowhead indicates the staining signals. Cy, cyst; Fc, follicle cell; Og, oogonia; PO, primary oocyte.

Fig. 6. The fates of the cells surrounding the oocytes are mediated by oocyte size. To identify the types of surrounding cells, we used serial sections to analyze the expression of Figla, Sertoli cell marker (Dmrt1), and follicle cell marker (Cyp19a1a). Figures **a**, **b**, and **c** are the serial sections. Figures **d**, **e**, and **f** are the serial sections. **a** and **d**) IHC of Figla. **b** and **e**) IHC of Cyp19a1a. **c** and **f**) IHC of Dmrt1. **g**) The relationship of oocytes size with the expression pattern of Figla, Cyp19a1a, and Dmrt1. Robust and slight stainings of Figla was classified according to staining strength, the reference point of Figla expression is the staining density at the at early primary oocytes (25 μm diameter). High expression was identified as "robust expression" and low expression was identified as "slight expression". An arrowhead indicates Dmrt1 expression. Asterisks indicate significance using a Student *t*-test ($P < 0.05$).

Fig. 7. *figla* expression is not influenced by sex steroids. Fish with primary oocytes with a diameter over 30 μm (with light expression of Figla) were selected for the experiment. **a**) Fish were given 17β-estradiol (E2, 1.5 μg/g of body weight) and methyltestosterone (MT, 1 μg/g of body weight) by intraperitoneal injection at stage 4 on Days 1, 3, and 5 (three injections in total). Ovarian tissue from the ovotestis was collected for analysis 24 h after the third injection (Day 6). Relative expression was normalized to the highest *figla* expression group (as 100%). **b**) AI (20 mg/kg of feed) and E2 (6 mg/kg of feed) were given to the fish from 3 mo (stage 2, Supplemental Fig. S1) to 8 mo until the oocytes size was larger than 30 μm (age, 8 mo). qPCR results of *figla* in the AI- and E2-fed fish was normalized to the highest expression group (as 100%). No differences were found among the experimental groups.

Fig. 8. Knockdown of *dmrt1* in the testis cannot change Sertoli cells to follicle-like cells. Partly *dmrt1*-deficiency in fish was produced by RNAi according to the previous study [17]. Gonad samples were collected 3 mo after the last injection (stage 3). Knockdown of *dmrt1* expression could not change the germ lines into the female fate. IHC analysis of Dmrt1 was observed in the control group (**a**), and no Dmrt1 signals were observed in the knockdown group (**b**). IHC analysis of Cyp19a1a showed that it was located in the interstitial cells in the control group (**c**) and the knockdown group (**d**).

A.


LT group (9 mo)
(E2 and AI treatment periods)
ST group (3 mo)
(E2 and AI treatment periods)
Control group
OT
OC
RT
TT
0 Mar
1 Apr
2 May
3 Jun
4 Jul
5 Aug
6 Sep
7 Oct
8 Nov
9 Dec
10 Jan
11 Feb (age)
Hatching
Gonad diff.
1st spawning season

B.

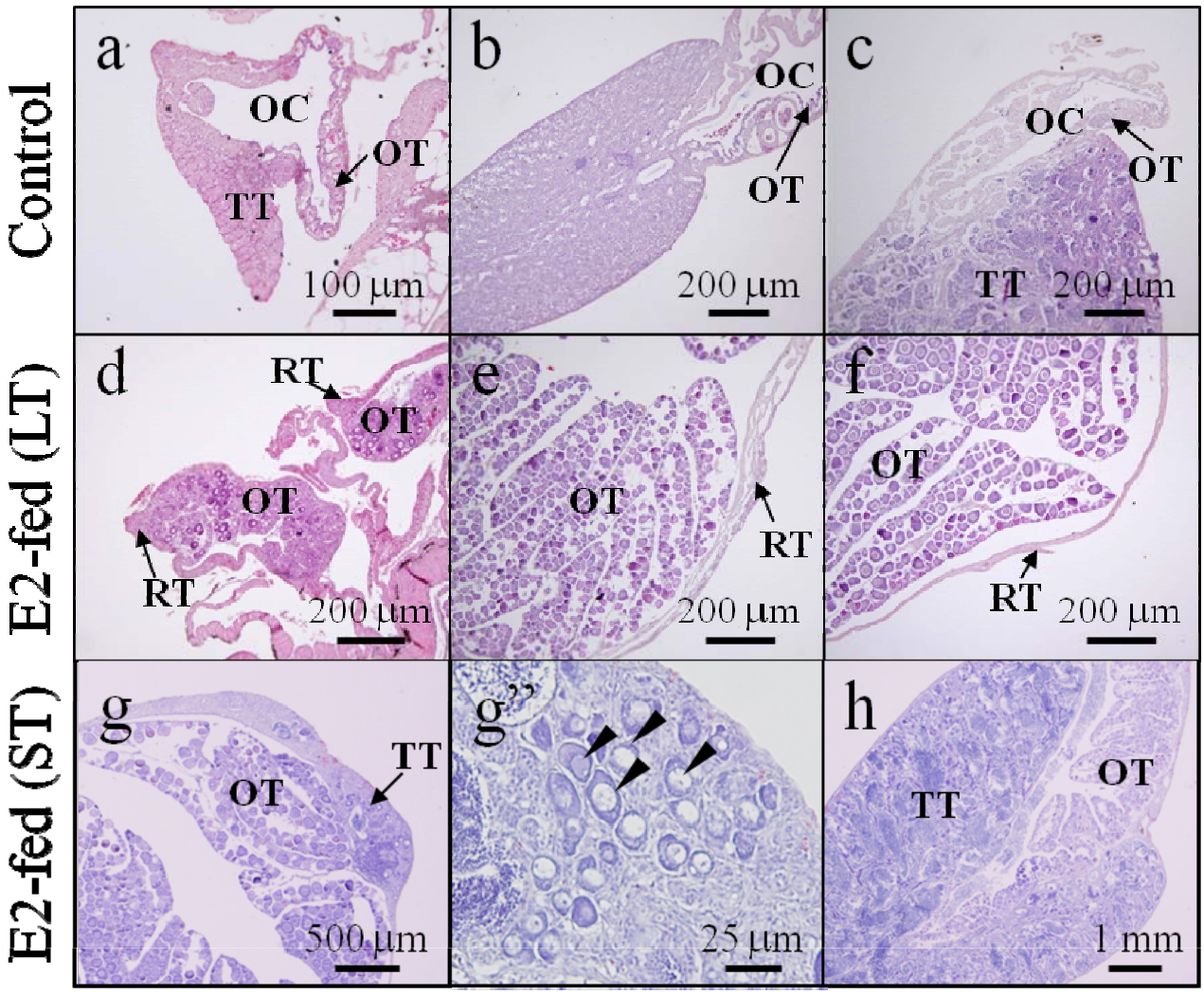

Control
E2-fed (LT)
E2-fed (ST)
OC
OT
TT
RT
100 µm
200 µm
500 µm
25 µm
1 mm

Fig. 1

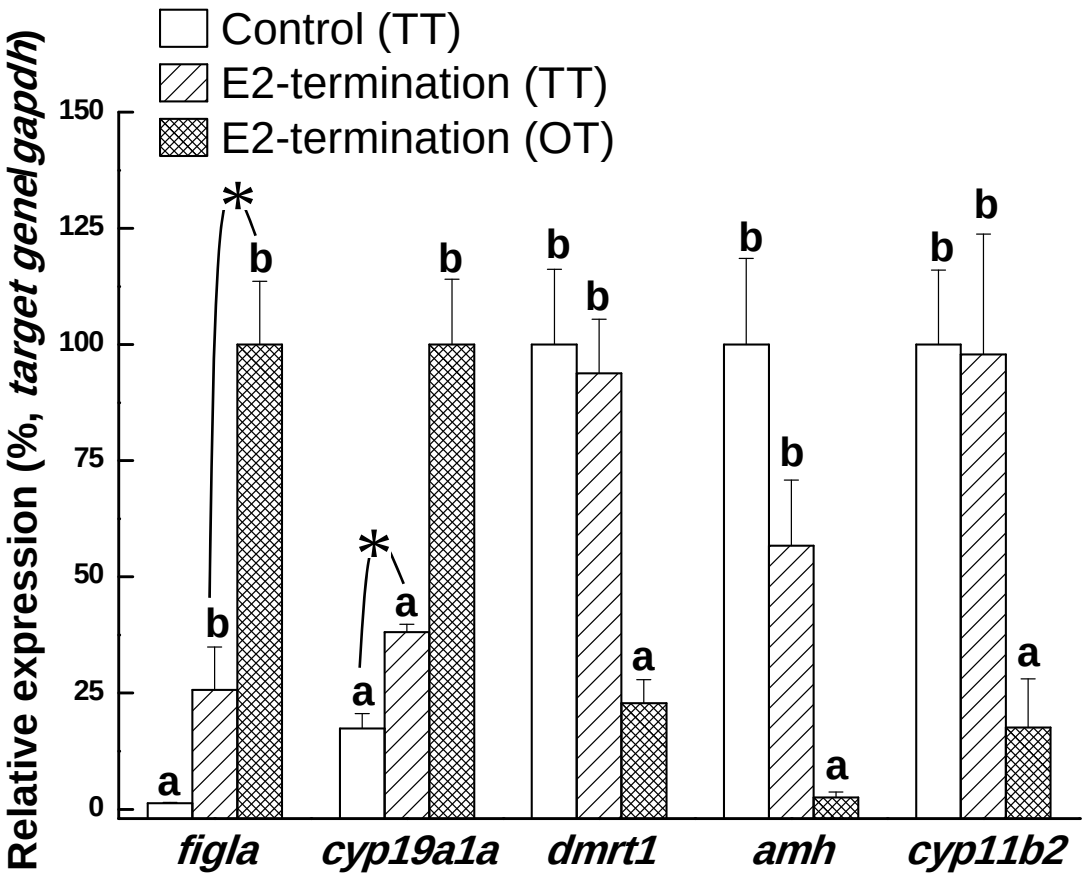

Control (TT)
E2-termination (TT)
E2-termination (OT)
Relative expression (%, target gene/gapdh)
150
125
100
75
50
25
0
figla
cyp19a1a
dmrt1
amh
cyp11b2
a
b
*

Fig. 2

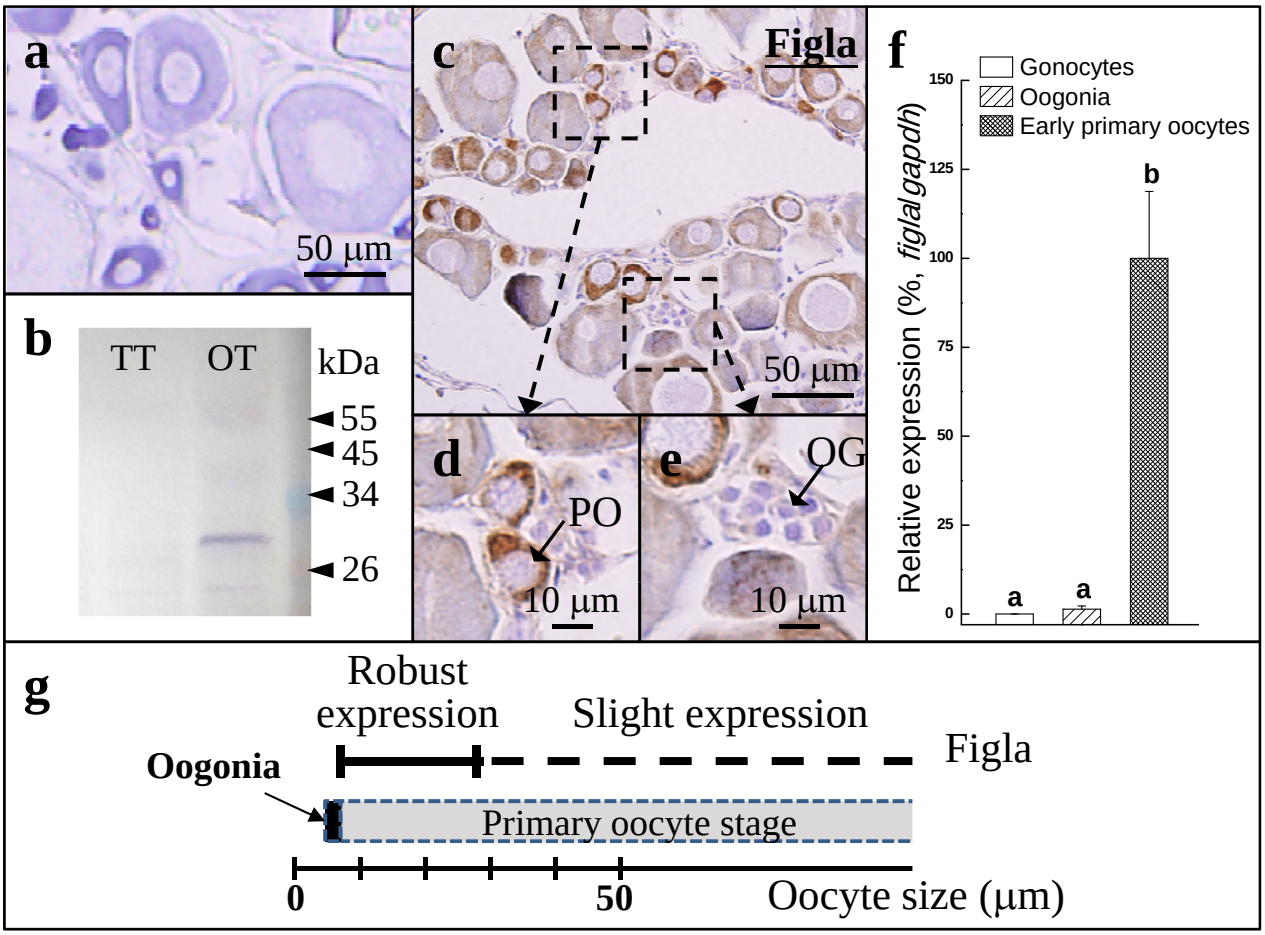

a
50 μm
b
TT
OT
kDa
55
45
34
26
c
Figla
50 μm
d
PO
10 μm
e
OG
10 μm
f
Gonocytes
Oogonia
Early primary oocytes
Relative expression (%, figla/gapdh)
150
125
100
75
50
25
0
a
a
b
g
Robust expression
Slight expression
Figla
Oogonia
Primary oocyte stage
0
50
Oocyte size (μm)

Fig. 3

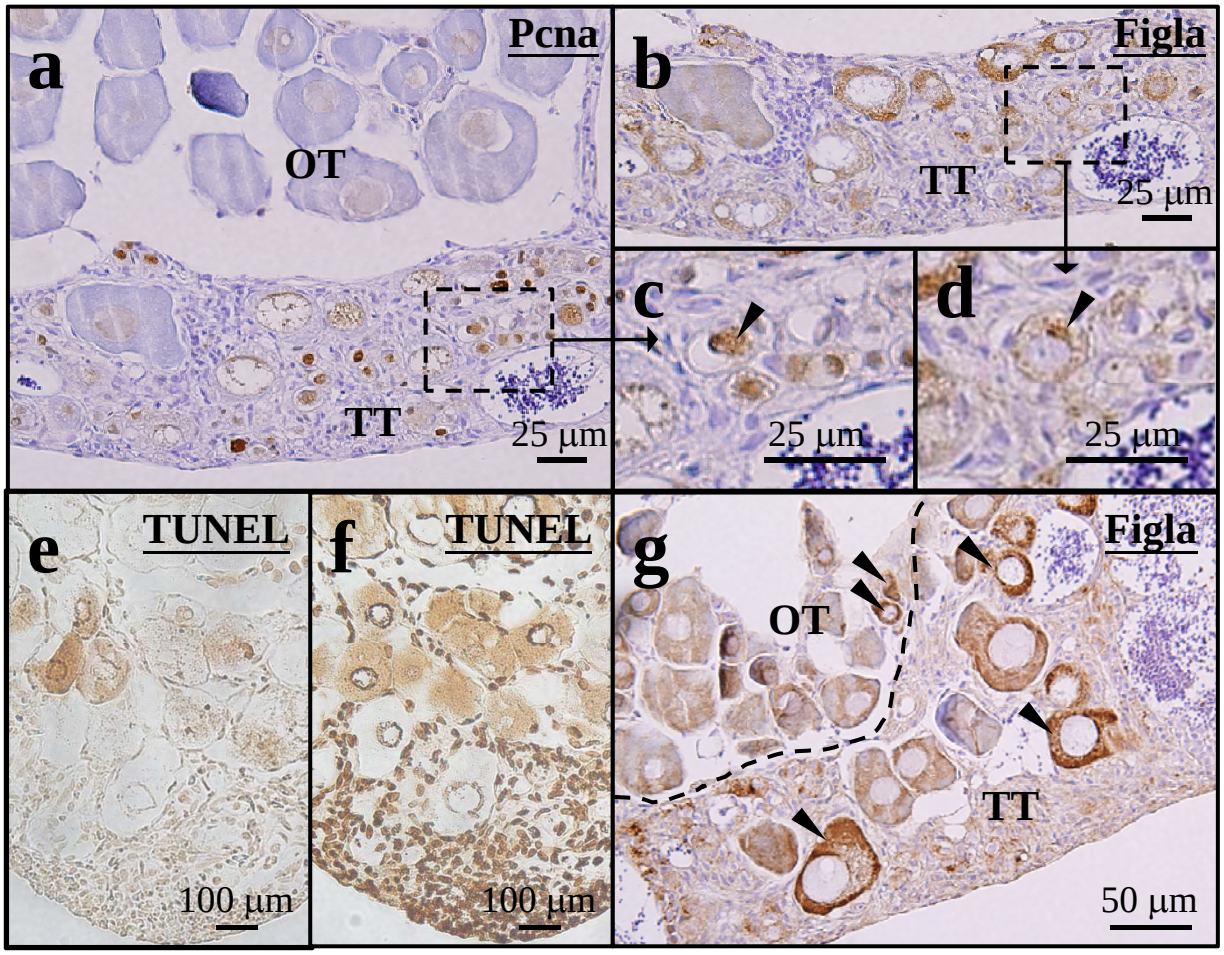

a
Pcna
OT
TT
25 µm
b
Figla
TT
25 µm
c
25 µm
d
25 µm
e
TUNEL
100 µm
f
TUNEL
100 µm
g
Figla
OT
TT
50 µm

Fig. 4

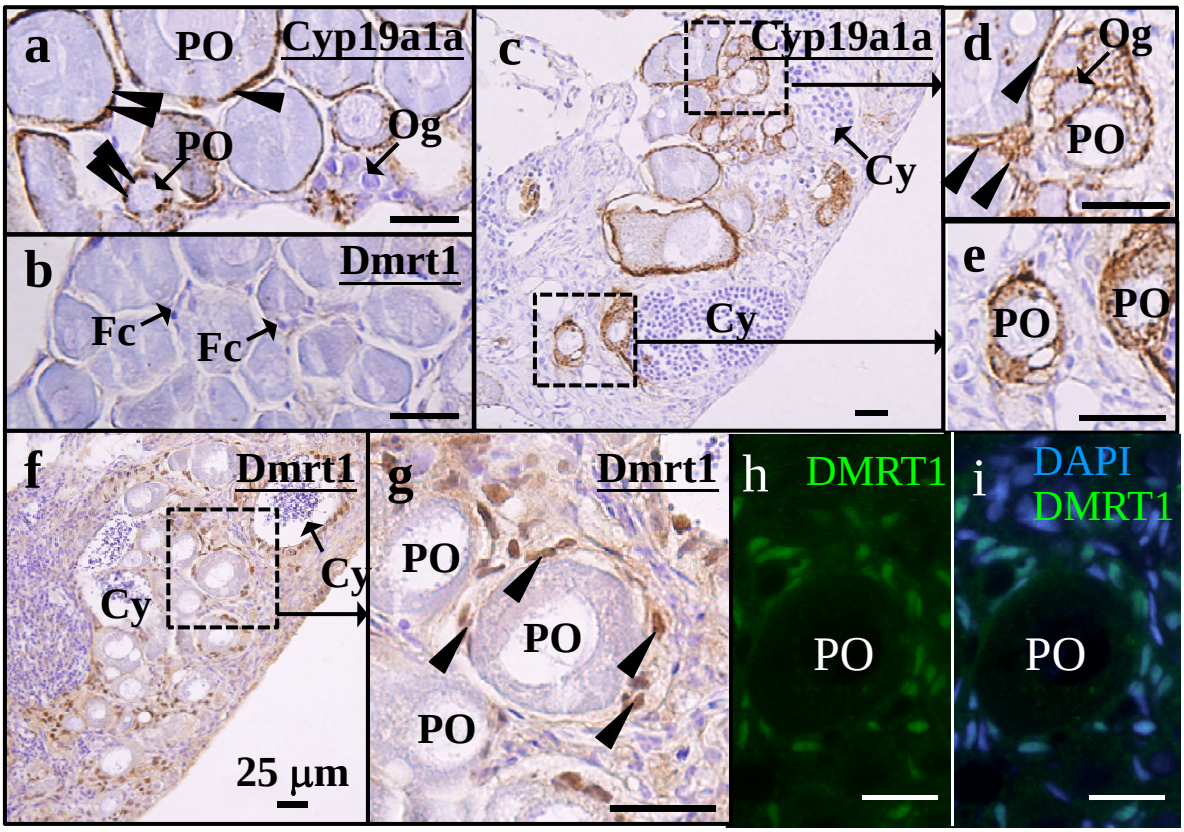
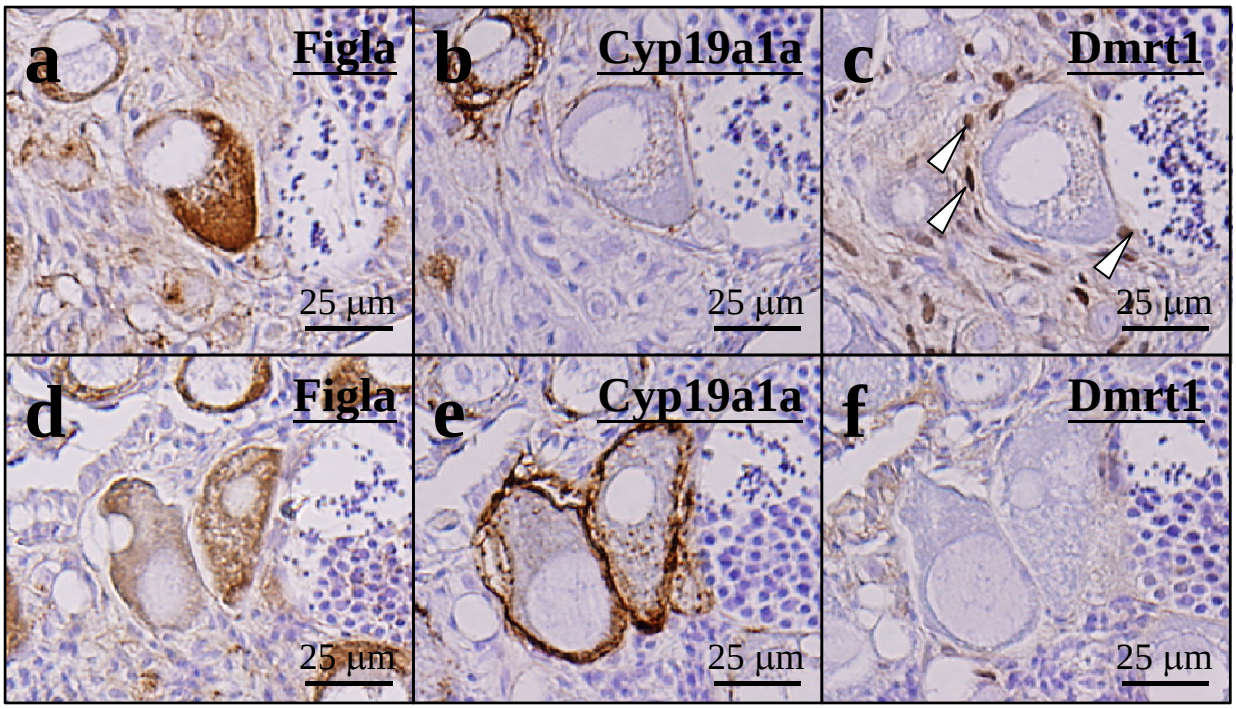

a
PO
Cyp19a1a
PO
Og
b
Dmrt1
Fc
Fc
c
Cyp19a1a
Cy
Cy
d
Og
PO
e
PO
PO
f
Dmrt1
Cy
Cy
25 μm
g
Dmrt1
PO
PO
PO
h
DMRT1
PO
i
DAPI
DMRT1
PO

Fig. 5


a
Figla
25 μm
b
Cyp19a1a
25 μm
c
Dmrt1
25 μm
d
Figla
25 μm
e
Cyp19a1a
25 μm
f
Dmrt1
25 μm

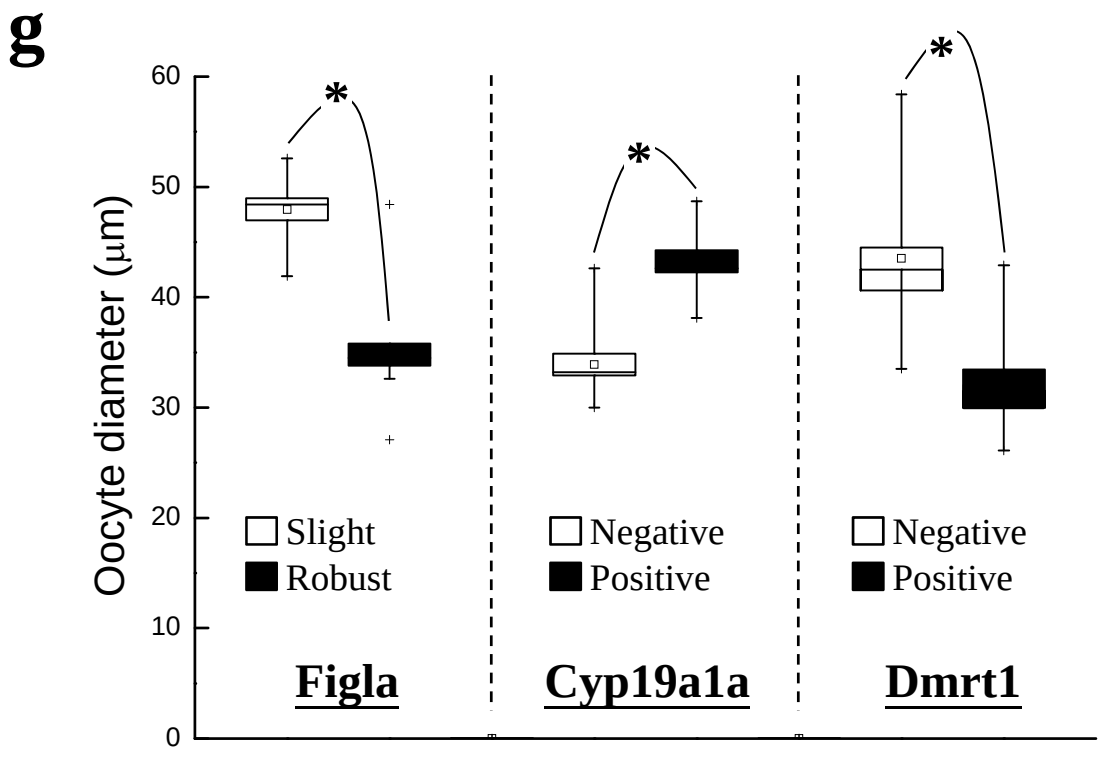

g
Oocyte diameter (μm)
0
10
20
30
40
50
60
*
*
*
Slight
Robust
Negative
Positive
Negative
Positive
Figla
Cyp19a1a
Dmrt1

Fig. 6

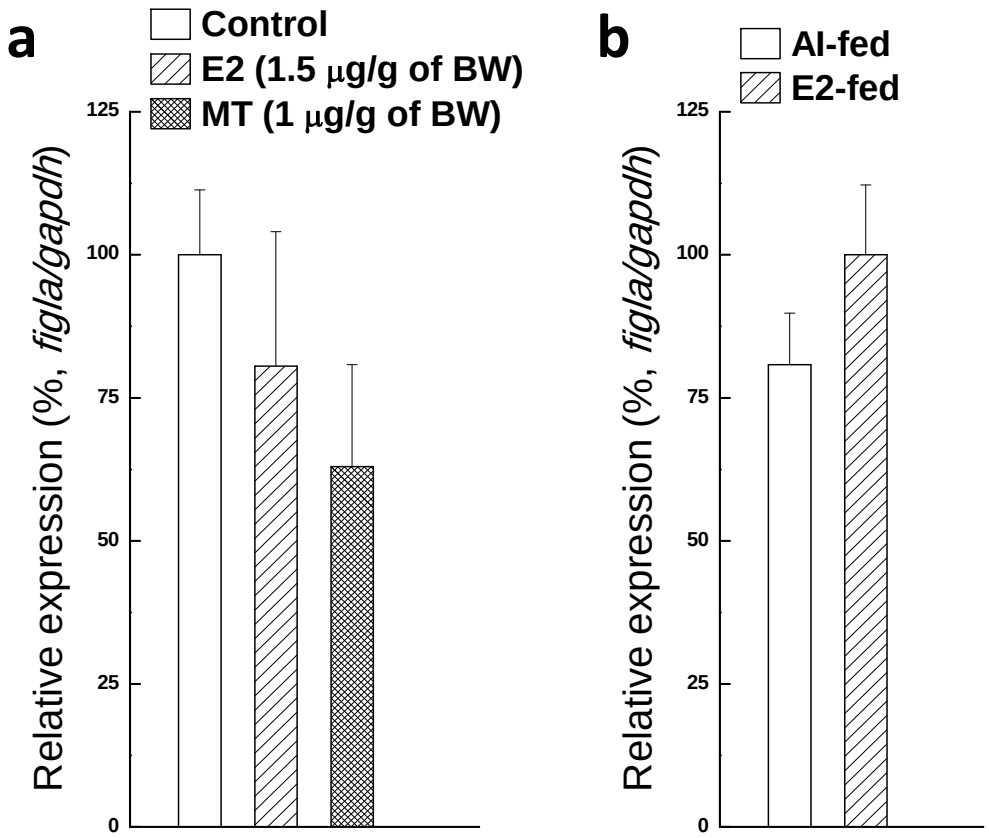

a
Control
E2 (1.5 μg/g of BW)
MT (1 μg/g of BW)
Relative expression (%, figla/gapdh)
125
100
75
50
25
0
b
AI-fed
E2-fed
Relative expression (%, figla/gapdh)
125
100
75
50
25
0

Fig. 7

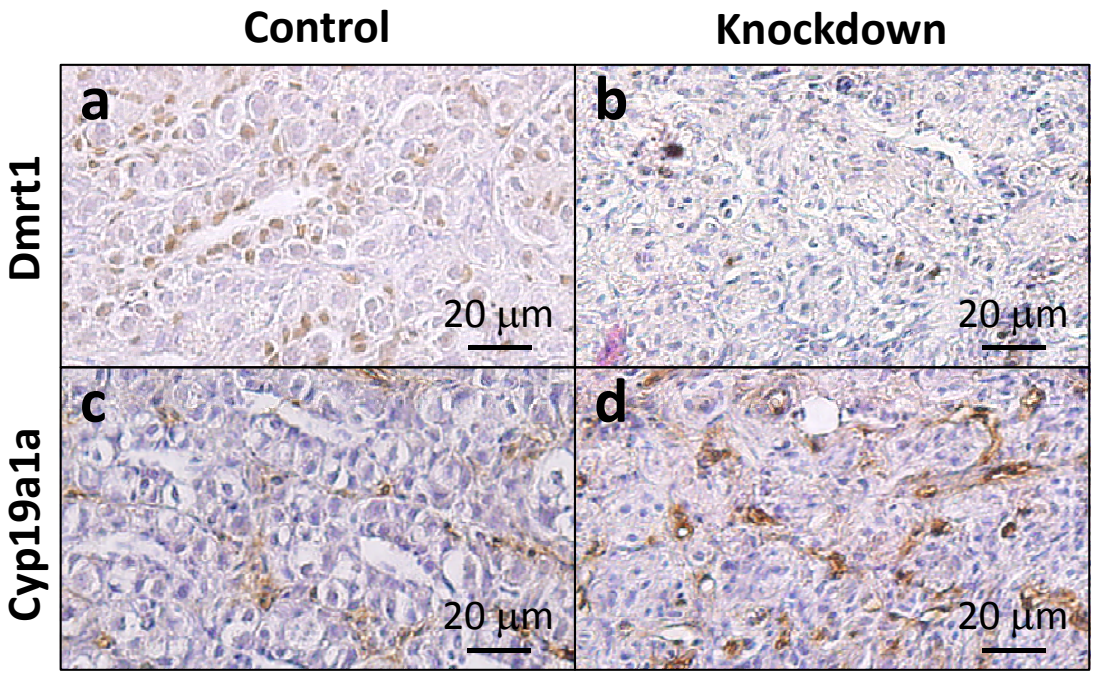


Fig. 8