Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists

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The sexual plasticity of the gonads is not retained after the completion of sex differentiation in vertebrates, except in some hermaphroditic species. Here, we report that the depletion of estradiol-17β (E2) by aromatase inhibitors (AI) for up to six months resulted in a functional female-to-male sex reversal in sexually-mature adults of two gonochoristic fish species, Nile tilapia and medaka. The sex-reversed fish showed a typical male pattern of E2 and androgen levels, secondary sexual characteristics, and male-like sex behavior, producing fertile sperm. Conversely, co-treatment of E2 inhibited AI-induced sex reversal. In situ hybridization of medaka gonads during AI-induced sex reversal indicated that cysts on the dorsal side of the adult ovaries are the origin of germ cells and Sertoli cells in the newly formed testicular tissue. Gonochoristic fish maintain their sexual plasticity until adulthood and E2 plays a critical role in maintaining the female phenotype.

Vertebrates have various mechanisms of sex determination, from genetic to environmental, but they all seem to have a neutral stage during embryonic development where the gonad is bipotential, and subsequently follows a sex differentiating pathway oriented towards either ovary or testis development. A large number of studies in fishes as well as other higher vertebrates suggest that during the period of sex differentiation, treatment with exogenous sex steroids such as estrogens or androgens causes sex reversal, but these steroids are effective only in early juveniles whose gonads are not sexually differentiated1. With the exception of various hermaphroditic fishes, reptiles and amphibians, the general consensus until recently was that gonochoristic vertebrates completely lose their sexual plasticity after differentiation of the gonad into either ovary or testis. However, a recent paper2 published during the progression of this research showed that type A spermatogonia isolated from cryopreserved whole testes of rainbow trout (Oncorhynchus mykiss) were able to differentiate into functional eggs and sperm. It is also of interest to note that in mice loss of the FOXL2 transcription factor in adult females (XX) by removing estrogens through in vivo inhibition of aromatase, the terminal enzyme responsible for E2 production, with highly specific aromatase inhibitors (AI). We used...
fadrozole (Fd) for tilapia and exemestane (EM) for medaka. The efficacy of Fd and EM in the induction of testicular differentiation in embryos of several non-mammalian vertebrates has already been proven. Adult breeding females of tilapia (one year old) and medaka (5 months old) were exposed to Fd and EM, respectively, until complete sex-reversal was achieved (6 months in tilapia and 2 months in medaka). The tilapia were fed a diet mixed with 200 μg/g Fd, while for medaka, 100 μg/L EM was added to the water in which the fish were reared. Very intriguingly, our results revealed for the first time in any vertebrate species that both tilapia and medaka females retain their sexual plasticity even in the adult stage. Furthermore, the present data indicate that estrogens are vital to the maintenance of female phenotype in the gonochoristic species.

Results

**E2 depletion induces male-specific gonadal phenotype in adult genetic female tilapia.** In all adult breeding females of tilapia treated with AI alone, plasma levels of E2 were significantly lower than those of the control groups (Fig. 1a and c). In contrast, no discernible changes were seen in the levels of 11-KT in fish at 60 (data not shown) and 90 days of treatment (dot) (Fig. 1b). Significant increases in plasma levels of 11-KT were observed in female tilapia at 180 dot (Fig. 1d). In contrast, the plasma E2 and 11-KT levels in fish with co-treatment of E2 were comparable to those of the controls (Fig. 1a to d).

Ovaries of exposed tilapia were analyzed periodically to detect testicular tissue. Gonads of untreated- and vehicle-controls contained numerous postvitellogenic follicles (untreated control, Fig. 1e and f; vehicle, data not shown). There was no indication of an extensive morphological change until 90 dot in ovaries of tilapia, although the degeneration of some vitellogenic follicles (Fig. 1g) and the appearance of small spermatogonial cysts were apparent. By 180 dot, almost all AI-treated fish had sex-reversed gonads with spermatogenic germ cells occupying either the entire or at least one-half of the gonads (Fig. 2a). Ten out of 20 females underwent complete sex reversal, and exuded sperm upon gentle pressure on the abdomen. In the sex-reversing fish, spermatogenic germ cells first appeared in the postero-ventral portion of the gonad, opposite to the main blood vessel. The ovarian cavity was situated in between the blood vessel and the newly-appeared spermatogenic cells. These fish had mature sperm and newly formed efferent ducts in the testicular region of their gonads; however, the ovarian cavity, an important characteristic feature of the female gonad, did not disintegrate upon sex reversal. Female tilapia receiving co-treatment of AI and E2 had ovaries with follicles at different stages of development comparable to those in the control ovaries (Fig. 2b).

![Figure 1](https://www.nature.com/scientificreports)
E2 depletion induces Leydig cell differentiation, male-specific secondary sex characteristics and sexual behavior in adult genetic female tilapia. Fish have two forms of aromatase: brain- and ovarian-form12–14. Ovarian-form of aromatase (Cyp19a1) catalyzes rate limiting steps of estrogen production in the ovary, and is shown to play a critical role in female sex differentiation via estrogen production13,14. Real-time PCR analyses revealed a marked reduction in cyp19a1 mRNA levels in the gonad after AI treatment, while gonads receiving co-treatment of AI with E2 had cyp19a1 mRNA levels comparable to those in the control gonads (Fig. 2c). We also examined changes in the Cyp19a1 expression in sex reversed gonads immunohistochemically. Strong signals of Cyp19a1-immunoreactive cells were observed in the control gonads (data not shown), whereas, in the sex-reversed fish, the signals were observed only in the inter-oocyte stromal tissue or the granulosa cells surrounding intact oocytes (Figs. 3a). No Cyp19a1 immunoreactive signals were observed in the testicular portion of the sex-reversed gonad. Interestingly, the interstitial cells in the testicular portion were positive for the steroidogenic enzymes such as cytochrome P450 cholesterol side-chain cleavage enzyme (SCC, Cyp11a1) and Δ5-3β-hydroxysteroid dehydrogenase (3β-HSD) (Fig. 3b and c). The granulosa cells of the intact follicles did not show any positive signal for 3β-HSD. DMRT1 is a good Sertoli cell marker in tilapia15. Real-time PCR analyses revealed a marked increase in the expression of dmrt1 in AI-treated gonads, while no such increase was observed in gonads receiving co-treatment of AI with E2 (Fig. 2d).

E2 depletion induces male-specific behavior and culminates in the development of a functional testis with viable sperm in adult genetic female tilapia. Throughout the experiment period, control females did not show any behavioral changes (Fig. 3d), while AI-treated females started showing male-specific chasing and territorial behavior along with the development of pink coloration on the head and extremities of the fins within a month of treatment like the males (Fig. 3e). Occasionally, semi-lethal fighting was also observed within the AI-treated group, where dominant fish knocked the abdomen of subordinates and eroded scales by biting on the lateral body surface. Furthermore, sex-reserved female tilapia made nests in the sand at the bottom of the fish tanks. When the sex-reversed XX fish was kept with normal XX females, the normal females were found to be carrying fertilized eggs in their mouths. In order to confirm the production of fertile sperm in AI-treated XX testes of tilapia, eggs from normal females were inseminated with sperm collected from the treated fish. The eggs were successfully fertilized and the resulting progeny included only females.

E2 depletion induces male-specific secondary sex characteristics in adult genetic female medaka. Further, we used adult breeding genetic females of medaka to confirm the effects of AI treatment on female phenotype. Unlike tilapia, genetic sexing is possible in medaka because the genetic males of this species possess the sex-determining gene, dmy, on the Y-chromosome, while the genetic females lack it. Moreover, expression of dmy has been found to be
unaltered by hormone treatments\textsuperscript{15,16}. Mature genetic females of medaka were paired with mature genetic males for breeding and the females that have bred for at least 1–2 weeks were exposed to AI. Soon after the onset of AI-treatment, medaka females stopped spawning and urogenital papillae started to shrink. At 10 dot, development of papillar processes, a male-specific secondary sexual characteristic (Fig. 4c), was observable on the anal fins of treated medaka females (Fig. 4e). Control females did not show development of such papillar processes on their anal fins (Fig. 4d).

Because of the difficulties in drawing sufficient amount of blood from medaka, we measured the tissue levels of E2 in the ovaries of the exposed fish. Control fish treated with the vehicle (99% ethanol) showed more amount of E2 in the ovaries, while AI-treated ovaries had an amount equal to that in the testis of the normal males (Fig. 4a) at 10 dot. 11-KT was significantly increased in the exposed ovaries at 10 dot when compared with that of the control ovaries, but was lesser than that in the testis of the normal males (Fig. 4b).

E2 depletion induces male-specific gonadal phenotype in adult, genetic female medaka. The ovaries of medaka prior to AI treatment and control fish contained numerous pre-vitellogenic and vitellogenic follicles together with some post-ovulatory follicles indicating that the fish were ovulating normally (Fig. 5a and b). In the medaka females, it took only 2 months for complete sex reversal of the gonads (Fig. 5c). At 60 dot, the exposed medaka females displayed testicular tissue with various stages of spermatogenesis (complete sex change, 8 fish; partial, 28 fish; none, 4 fish).

Connective tissue-like structures with some kind of glandular cells were seen in the central part of the gonads. Some of these cells were steroidogenic because they showed the expression of SCC (Fig. 5d).

The secondary sex characters, sex behavior and fertility of the sex-reversed fish were analyzed. Medaka females have a small anal fin, while males show a large anal fin and a forked dorsal fin (Fig. 6a). The exposed females displayed typical male secondary sex characters, long and forked dorsal fin and large anal fin, at 60 dot (Fig. 6b and c). However, the fish remained white without any orange-red pigmentation. Upon pairing with normal females, they displayed male-like sex behavior such as chasing the females, crossing the females, positioning under the females and dancing in circles to attract the attention of the females (Supplementary videos 1–4). However, the females never showed an interest towards these sex-changed white males. Thus, normal mating was not possible. In order to confirm the production of fertile sperm in the XX testes of AI-treated medaka, eggs from normal females were inseminated with sperm collected from the treated fish. The eggs were successfully fertilized (Fig. 6d) and the genotype of F1 progeny was confirmed by genomic PCR using primers for \textit{dmy} and \textit{dmrt1} (Data not shown). Their genomes were free of \textit{dmy}. They exhibited normal development and fertility, producing progeny in normal sex ratios like any other normal females (Fig. 6e).

\textbf{Germline cysts along the germinal epithelium are the origin of spermatogonia and Sertoli cells.} We then examined the origin of the testicular tissue in AI-treated XX fish by exposing medaka to EM for
shorter periods. It was previously reported that SRY-box containing gene 9b (sox9b)-expressing cells in medaka later exhibited dmrt1 in XY gonads and forkhead box L2 (foxl2) in XX gonads during sexual differentiation, indicating that the sox9b-expressing cells were the precursors of both testicular Sertoli and ovarian granulosa cells17. More recently, we showed that a new factor, gonadal-soma derived factor (gsdf), is also expressed in Sertoli cells and is associated with testicular differentiation18. Unlike dmy, the expression of gsdf is closely linked to the gonadal phenotype18,19. As described above, the ovaries of fish prior to AI treatment and control fish contained numerous pre-vitellogenic and vitellogenic follicles. Further, control ovaries of medaka contained cysts with single or clusters of germ cells exhibiting characteristics of germ line stem cells (GSCs) in the germinial epithelium (GE) on the side of the ovary adjacent to the ovarian cavity (Fig. 7a to c). These cells expressed the homologue of the germ cell marker vasa (olvus in medaka) and the somatic cells around these germ cells were sox9b-positive and gsdf-negative, indicating that the somatic cells were undifferentiated (Fig. 7c and d). At 30 dot, these cysts were found to have expanded in size, taking on the appearance of a spermatogonial cyst (Fig. 8a and b). Importantly, sox9b-expressing somatic cells surrounding these germ cells were starting to express gsdf strongly, indicating the initiation of Sertoli cell differentiation (Fig. 8c and d). At 40 dot, advanced stages of spermatogenesis were observable along the GE, with strong signals of gsdf in the somatic cells (Fig. 8e and f).

In medaka, gonads at 30 dot contained not only spermatogonial cysts, but also atretic follicles (Fig. 8g). Although foxl2 was expressed in these atretic follicles, neither granulosa cell (cyp19a1) nor Sertoli cell (gsdf) markers were detected (Fig. 8h to j). Similarly, sox9b was also not found (Fig. 8k). None of these genes were expressed in atretic follicles even at advanced stages of degeneration.

**Discussion**

Until recently, most vertebrates were believed to have lost sexual plasticity after the terminal differentiation of their gonads and remain the same sex throughout their life spans. Studies done in adult mice by conditional abrogation of Foxl23 and Dmrt14 have challenged this dogma as the somatic cells in the gonads of these animals underwent transdifferentiation. Similarly, although AIs have also been proved successfully in demonstrating the critical role of estrogens in the induction of ovarian differentiation during the period of sexual differentiation, these inhibitors have never been shown to induce sex reversal in any adult vertebrate species. Therefore, in this study, we used two different AIs, Fd and EM, to block the conversion of androgens to estrogens and examined whether lack of estrogen can reverse the gonadal morphology in two adult, sexually-mature gonochoristic species. Interestingly, we found that both non-steroidal and steroidal AIs were effective in blocking estrogen production and induced a functional sex reversal from females to males in tilapia and medaka. Our results have demonstrated that the AIs are sufficient to induce not only the testicular structure, but also the phenotypic transformation including sexual behavior. Our data, for the first time in any vertebrates, has shown that sexual plasticity is preserved even in adulthood.

The first obvious signs of morphological changes in XX gonads of tilapia and medaka seen after the onset of AI treatment were the
degeneration of female germ cells. The specific action of AIs on these morphological changes was confirmed by another AI group of tilapia co-treated with E2. Plasma E2 levels of these fish were comparable to those of controls. Co-treatment of E2 could rescue the germ cells of these fish from degeneration, proving the action of AI to be specific. It is interesting to note that the initial stage of AI-induced sex reversal in tilapia appears to be analogous to the events that follow the natural sex reversal from female to male in a hermaphroditic fish, the

Figure 5 | Gonadal morphology of XX medaka. (a) Ovarian morphology of control XX medaka showing vitellogenic and post-vitellogenic follicles together with post-ovulatory follicles (black arrow). (b) Magnified image of the post-ovulatory follicle shown in the dotted rectangle area in (a). (c) Testis formed in an adult breeding XX medaka by inhibition of aromatase for 60 days. Scale bars are 500 μm (a), 250 μm (b), 80 μm (c) and 50 μm (d).

Figure 6 | Fin structures and fertility assessment of sex-reversed XX medaka. (a) Fin morphology of the control XX female medaka at 60 dot. Small anal and unforked dorsal fins are shown. (b) Fin morphology of an XY male medaka. Anal fin is large and dorsal fin is forked. (c) Fin morphology of a sex-reversed XX male medaka after 60 days of AI treatment. Anal fin is large and dorsal fin is slightly forked. (d) Four days old embryos generated by artificial insemination of the eggs from a donor XX female with the sperms from the sex-reversed XX medaka. All embryos were of XX genotype. (e) One of the XX females (shown in (d)) of the F1 progeny carrying fertilized eggs (dotted circle). The authors thank Dr. Bindhu Paul-Prasanth for capturing this photograph.
Figure 7 | Germline stem cells on the germinal epithelium of the medaka ovary. (a) A germline stem cell (black arrow) having the morphology of a primordial germ cell on the germinal epithelium adjacent to the ovarian cavity (OC) in the control ovary of an adult XX medaka. An oocyte (Oy) is also seen. (b) GSC and the surrounding somatic cells in the ovary of a control XX medaka showing expressions of olvas (red) and sox9b (green), respectively. Ovarian cavity (OC) is seen. (c) Digitally magnified image of the area within the dotted rectangle in (b). The GSC shows the olvas (red) expression, while the somatic cells around the GSC show sox9b (green) expression. Nuclei are stained with DAPI (blue) in (b) and (c). (d) Somatic cells (dotted lines and arrowhead) around the GSCs hybridized with amplified RNA probe for gdf. No expression was detectable. The section is counter-stained with hematoxylin-eosin. Scale bars are 80 μm (a), 50 μm (b), 50 μm (c) and 5 μm (d).

Figure 8 | Origin of Sertoli cells and spermatogonia in sex-reversed XX medaka. (a) A spermatogonial cyst in the ovary of an AI-treated XX medaka at 30 dot. An oocyte (Oy) and ovarian cavity (OC) are seen alongside. (b) Magnified image of the area within the dotted rectangle in (a). Cyst shows proliferated germ cells having morphology similar to that of type A spermatogonia. (c) Somatic cells around the germ cells in spermatogonial cysts in AI-treated medaka showing sox9b (green) expression at 30 dot. Nuclei are stained with DAPI (blue). (d) Somatic cells around the germ cells in spermatogonial cysts in AI-treated medaka co-expressing gdf (orange) at 30 dot. Images shown in (c) and (d) are of the same section hybridized with amplified RNAs of sox9b and gdf. Nuclei are stained with DAPI (blue). (e) gdf expression in the somatic cells around the spermatogenic tissue in sex-reversed XX medaka at 30 dot. Ovarian cavity (OC) is seen near to the spermatogenic tissue. (f) Spermatogenic tissue with spermatocytes and spermatids in the gonads of AI-treated XX medaka located near to the ovarian cavity (OC). (g) A young atretic follicle stained with hematoxylin-eosin in the ovary of an AI-treated XX medaka at 30 dot. (h) An atretic follicle hybridized with aRNA probe of cyp19a1at 30 dot. No expression was detectable. (i) An atretic follicle showing expression of foxl2 at 30 dot. (j) An atretic follicle hybridized with aRNA probe of gdf at 30 dot. No expression was detectable. (k) An atretic follicle hybridized with aRNA probe of sox9b at 30 dot. No expression was detectable. Scale bars are 50 μm (a), (c), (d), 10 μm (b), (e), (f) and...
protogynous wrasse, Thalassoma duperrey. In wrasse, the initial stage of sex reversal was accompanied by a decrease in plasma levels of E2, proving that E2 is an important trigger of sex reversal from the female to male phenotype in both hermaphroditic and gonochoristic fishes. Thus, once estrogen levels decrease below their physiological threshold, germ cells in female fish do not seem to receive proper nourishment and undergo degeneration, suggesting that estrogen is critical for the maintenance of the ovary in adults. Lately, importance of estrogen in the maintenance of ovarian phenotype was demonstrated in mice also through conditional abrogation of the gene Foxl2. Ovaries of these mice had a drop in serum E2 levels and a concomitant rise in the androgen, leading to the degeneration of oocytes and induction of testicular cord formation.

In the present study, the degeneration of female germ cells was followed by the appearance of testicular tissue. Since numerous studies on fish including tilapia and medaka have shown that treatment of XX larvae with androgens induced sex reversal and testicular development, it is possible that endogenous androgens play a role in the first appearance of testicular tissue in AI-treated gonads. However, this does not appear to be the case in tilapia, because there were no significant increases in plasma levels of 11-KT in female tilapia during early stages of AI-induced sex reversal. The gonadal histology of these fish revealed that the newly-developed testicular tissue was in the early stages of development and further, upon maintenance for longer term, the testicular portion of these fish had mature sperm. We could also find that the interstitial cells in the testicular region of gonads of AI-treated tilapia were steroidogenically active as they were positive for SCC and 3β-HSD, of which the latter denotes the presence of Leydig cells in the testicular region. Accordingly, there was a significant rise in the plasma levels of 11-KT in the latter fish, suggesting that androgens are probably required only for the later stages of spermatogenesis. No direct experimental evidence has been provided till date to prove the role of endogenous androgens in initial testicular differentiation in fish. Previous studies have shown a correlation between the timing of the initiation of spermatogenesis and up-regulation of the mRNA expression of the 4 steroidogenic enzymes including cytochrome P450 (CYP11B1) in XY tilapia. The study in the zebrafish, Danio rerio also found that the expression of 11β-hydroxylase (a steroidogenic enzyme essential for 11-KT synthesis) became highly expressed only when the testis was fully differentiated. Taken together, these findings indicate that androgens are essential for spermatogenesis, but not for initiation of testicular differentiation.

Almost all tilapia treated with Fd for 180 days and medaka treated with EM for 60 days had sex-reversed gonads with spermatogenic germ cells occupying either the entire or at least one-half of the gonads. These sex-changed fish were capable of displaying male-specific territorial and sex-behavior, indicating that the brains also have retained the sexual plasticity even into adulthood. Probably, the decline in the circulating levels of E2 and the rise in 11-KT levels in AI-treated fish induced the male-specific territorial and sex-behavior in these fish. However, mating did not occur in the case of medaka as the control females were not interested in the sex-reversed XX fish. These findings further suggest that abrogation of E2 in the adult gonochoristic species can reverse not only the gonadal development, but also the sexual identity of the individual concerned.

Our results of AI treatment in medaka provided important insights about the origin of the germ and testicular somatic cells in sex-reversed ovaries. It is worth mentioning that testicular tissue first appeared in the periphery region of the ovary. The control ovaries of adult tilapia and medaka contained some isolated cysts adjacent to the ovarian cavity. These cysts of medaka generally contained a single PGC-like vasa-positive germ cell which was surrounded by a few somatic cells. AI treatment induced proliferation of these germ cells in the exposed medaka ovaries, indicating that the PGC-like germ cells along the GE underwent de novo differentiation in the absence of E2, giving rise to the testicular germ cells in the AI-treated gonads. These findings indicate that the cysts on the dorsal side of the adult ovaries are the origin of germ cells in the newly formed testicular tissue. Very recently, such cysts in ovaries of medaka were identified as germinal cradles; some of these germ cells were demonstrated to be germline stem cells (GSC) capable of undergoing de novo differentiation into oocytes. The present study has further proven that GSCs in medaka are bipotential and can differentiate into spermatogonia and eventually into spermatozoa. Importantly, several studies in mammals have also identified GSCs from ovaries of adult females, indicating that the presence of GSCs in adult ovaries is a common phenomenon among the vertebrates. We have now shown that GSCs in medaka females possess the ability to differentiate into spermatogonia in the absence of estrogens.

Another important question is the source of other testicular cells such as Sertoli cells and Leydig cells which were proliferated in the gonad during AI-induced sex reversal. As described above, cysts seen in the control ovaries also contained a few sox9b-expressing somatic cells which surrounded the germ cell. Interestingly, these somatic cells of medaka became positive for gsdf after 30 days of AI treatment. Recently, we have found in medaka that gsdf was expressed exclusively in primordial gonads of only the genetic males at 6 dpf. When the XY embryos were treated with E2 or diethylstilbestrol, in order to reverse their phenotypic sex, a decline was observed in the expression of gsdf in the treated embryos. We have concluded from these results that GSDF plays an important role in earlier stages of testicular differentiation in medaka, probably down stream to DMY. Thus, it is possible that somatic cells present in cysts may be the origin of Sertoli cells in newly formed testicular tissue.

Recently, Uhlenhaut et al. reported that deletion of Foxl2 in adult ovaries of the genetically female mice leads to somatic cell reprogramming (transdifferentiation of ovarian granulosa cells to testicular Sertoli cells) and development of seminiferous tubule-like structures, although these gonads were devoid of germ cells. Moreover, loss of Dmrt1 in the adult testis caused the reciprocal sex transformation, from Sertoli cells to granulosa cells. In medaka, gonads at 30 dpf contained not only spermatogonial cysts, but also atretic follicles. Thus, it is possible that these atretic follicles are the potential sources of somatic cells in the newly formed testicular tissue. However, these cells did not show the markers for Leydig and Sertoli cells even at advanced stages of degeneration suggesting that transdifferentiation was not the mechanism by which sex-reversal was initiated in the gonads of medaka. The differences in the mechanisms by which sex reversal was induced in the present study and the two previous studies suggest that the hormonal and genetic pathways function differently, yet the interplay between them is vital for the maintenance of the sexual phenotypes.

Our observations have corroborated that steroid hormones play a critical role in regulating the developmental fate of bipotential GSCs and somatic cells around them in the ovaries of adult females. However, the possibility for reprogramming of somatic cells, especially Leydig cells, in the estrogen-depleted gonads remains to be investigated further. This is the first demonstration that individual adult vertebrates can produce gametes that they are not genetically programmed for even after the terminal differentiation of their gonads. Furthermore, this investigation has proven that E2 is critical not only for the maintenance of ovarian phenotype, but also for the maintenance of femaleness in sexually-reproducing organisms, emphasizing the importance of protecting wild life from increasingly getting exposed to the man-made therapeutic drugs and endocrine disruptors. If studies on mice demonstrate the roles of FOXL2 and DMRT1 in the prevention of postnatal sexual reprogramming of the gonads, our study underscores the requirement of sex hormones for overseeing the maintenance of the genetic program that establishes the sexuality of an organism. Finally, medaka may be an ideal model for studying germine stem cell biology in vertebrates.
Methods

Animals. Tilapia. Cichlid fish (Nile tilapia, Oreochromis niloticus) were used as the experimental animals. The entire population of genetically female (XX) fish were obtained by artificial fertilization of eggs from a normal female (XX) with the sperm from a sex-reversed pseudomale (XX). Fertilized eggs were cultured in small glass tubes with a constant supply of freshwater and aeration for 10 days. After hatching, larvae were transferred to 50 L glass aquaria, where they were reared until experimentally, under natural photoperiod. The fish were fed with commercial salmon food (Oriental, Japan) 2 times a day. All experiments using tilapia were carried out with the approval from the institutional ethics committee of the University of the Ryukyus, Japan, strictly following the guidelines set for the usage of animals by this committee.

Medaka. The Qur strain of medaka was used for this study. All sex reversal experiments were carried out using 5 months old adult breeding females. Mature females were paired with mature males for mating and spawning for 1–2 weeks before the onset of treatment. All the fish were maintained under laboratory conditions with 14 h light and 10 h dark cycles at 28–26 °C. The fish were fed with brine shrimp 3 times a day. All experiments using medaka were carried out with the approval from the institutional ethics committee of National Institute for Basic Biology, Japan, strictly adhering to the guidelines set for the usage of animals by this committee.

Treatment. Tilapia. Aromatase inhibitor (AI) (Fadrozole, Novartis Japan) was dissolved in 99% ethanol and added to salmon feed at a concentration of 200 μg/g feed. Only ethanol was added to the control feed. After drying the feed, it was refrigerated until further use. For the preparation of AI and ethanol containing feed, AI was dissolved in 99% ethanol and added to the salmon feed mixed with AI at 200 μg/g feed. Six months old females were divided into 3 groups (50 fish each). One group was fed with control feed and the other with AI feed at 200 μg/g concentration. The effective dose was selected from a previous experiment published elsewhere. The third group was fed with AI and ethanol (200 μg each) together. The treatments were continued with periodical examination of the gonads for signs of complete sex-reversal in the fish belonging to the Al group.

Histology, immunohistochemistry (IHC) and in situ hybridization (ISH). Tilapia. The ovaries were dissected out every month and fixed in Bouin’s reagent for histology and immunohistochemical examinations. Samples for histology and IHC were mounted in paraffin and the sections were cut at 7 μm thickness. Sections for histology were stained with hematoxylin–eosin according to standard protocols. Immunostaining was performed for Cyp11a1, 3β-HSD and Cyp19a1 in both control and treated gonads according to Kobayashi et al. Medaka. The gonads were dissected out every 10 days and cut into two parts along the mid-sagittal line. One part was fixed in Bouin’s reagent for histology and the other part was fixed using 4% paraformaldehyde (PFA) for IHC. Samples for histology, IHC and IH were mounted in paraffin and the sections were cut at 5 μm thickness using either manual (Leica Systems, Germany) or semi-automatic (Leica) microtomes. Sections for histology were stained with hematoxylin–eosin according to standard protocols. ISH was performed with digoxigenin (DIG)-labeled (Roche, Germany) probes for cyp19a1, fosl2, gdf5 and sox9b. Hybridization signals were detected using alkaline phosphate conjugated anti-DIG antibody (Roche) and NBT/BCIP as chromogen (Roche). Fluorescence two-color ISH was done for the detection of undifferentiated PGC-like cells using probes for sox9 and olvus following previously described methods (SS) (sox9b probe was labeled with DIG and olvus probe was labeled with fluorescein isothiocyanate (FITC). For detection of the signals, TSA Plus Fluorescein/TMR system (PerkinElmer, Inc., Waltham, MA) was used. Signals were assigned digitally to the hybridization signals of sox9/sox9b (green) and fosl2 (red)/df5 (orange) using Zeiss software provided by the manufacturer (Carl–Zeiss).

Hormone measurement. Tilapia. Plasma levels of E2 and 11-KT of tilapia were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described by Rahaman et al. Inter- and intra-assay variations were below 10%. Medaka. Because of the difficulties associated with drawing a sufficient volume of blood for the assays, levels of E2 and 11-KT in gonadal tissue were measured by ELISA according to the descriptions in Nash et al. Briefly, steroid hormones were extracted from the gonad samples with a standard ethyl ether extraction method and were diluted in 0.05 M borate buffer containing 0.5% BSA (assay buffer). Commercially available, purified E2 (Sigma Chemical Co., St. Louis, MO) was diluted serially from 4 to 0.078 ng/ml in assay buffer and used as standard samples (10 standards) to construct a standard curve. For the measurement of 11-KT, the antibody 11-Oxo-testosterone-3-CMO-BSA IgG (Cosmo Bio Co. Ltd.) was used. Commercially available 11-KT (Cosmo Bio Co. Ltd.) was used to generate the standard curve. All samples were tested in triplicate.

RNA isolation, reverse transcription (RT) and real-time PCR. Total RNA was extracted from ovaries and testes of tilapia with Isogen (Nippon Gene) following manufacturer’s instructions and then reverse transcribed into cDNA using 0.1 μmol of Oligo (dT)20 and 40 units of M-MLV reverse transcriptase (Invitrogen). The mRNA levels of cyp19a1 and dmrt1 were measured. Primers were designed in such a way that one primer of each primer pair was located on the exon-intron boundary to avoid the amplification of the genomic DNA. To quantify the copies of transcripts, cDNAs from gonads were subjected to quantitative real-time PCR (Applied Biosystems), which was performed in 20 μl reaction volumes, consisting of 6 ng template cDNA, 1× SYBR Green Universal Master Mix (Applied Biosystems), and 400 nM of primers.

Statistical analysis. Tilapia. All values were expressed as the mean ± SEM, and statistical comparisons were made between untreated and treated fish (n = 12) using Student’s t-test at 95% level of significance (SPSS 9.0). To compare significant differences between control, AI-treated and AI/E2-treated fish, one way ANOVA was used followed by Scheffe’s post hoc test (P < 0.05). Medaka. All values were expressed as the mean ± SEM (n = 5), and statistical comparisons were made among untreated female and treated female and male using Student’s t-test (Graphpad Prism) at 95% level of significance.

1. Devlin, R. H. & Nagahama, Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191–364 (2002).
2. Lee, S., Iwasaki, Y., Shikina, S. & Yoshizaki, G. Generation of functional eggs and sperm from cryopreserved whole testes. Proc. Natl. Acad. Sci. USA. 110, 1640–1645 (2013).
3. Uhlenhaut, N. H. et al. Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. Cell 139, 1130–1142 (2009).
4. Matsumi, T. et al. Dmy is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417, 559–563 (2002).
5. Nanda, I. et al. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Orzias latipes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 69–74 (2010).
6. Koffman, P., Münsterberg, A., Capel, B., Vivian, N. & Lovell-Badge, R. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348, 450–452 (1990).
7. Smith, C. A. & Sinclair, A. H. Sex determination: insights from chickens. BioEssay 26, 120–132 (2004).
8. Elbrecht, A. & Smith, R. G. Aromatase enzyme activity and sex determination in chickens. Science 255, 467–470 (1992).
9. Rukasana, S., Pandit, N. P. & Nakamura, M. Efficacy of exemestane, a new generation of aromatase inhibitor, on sex differentiation in a gonochoristic fish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 69–74 (2010).
10. Choudhokova, A. & Callard, G. V. Identification of multiple CYP19 genes encoding different cytochrome P450 aromatase isoforms in brain and ovary. Endocrinology 139, 2179–2189 (1998).
11. Holtzer, J. Y., McAndrew, B. J. & Penman, D. J. Cloning of brain aromatase inhibitor, on sex differentiation in a gonochoristic fish. Aquaculture 348, 450–452 (1990).
12. Smith, C. A. & Sinclair, A. H. Sex determination: insights from chickens. BioEssay 26, 120–132 (2004).
13. Elbrecht, A. & Smith, R. G. Aromatase enzyme activity and sex determination in chickens. Science 255, 467–470 (1992).
14. Rukasana, S., Pandit, N. P. & Nakamura, M. Efficacy of exemestane, a new generation of aromatase inhibitor, on sex differentiation in a gonochoristic fish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 69–74 (2010).
15. Nagahama, Y. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol. Biochem. 31, 105–109 (2005).
16. Suzuki, A., Nakamoto, M., Kato, Y. & Shibata, N. Effects of estradiol-17β on germ cell proliferation and DMY expression during early sex differentiation of the medaka, Orzias latipes. Aquaculture 222, 791–796 (2000).
17. Nakamura, S. et al. Sox9b/sox9a2–EGFP transgenic medaka reveals the morphological reorganization of the gonads and a common precursor for both the female and male supporting cells. Mol. Reprod. Dev. 75, 472–476 (2008).
18. Kadowaki, Y. et al. Expression of alternative modal soma derived factor (GSDV) is spatially and temporally correlated with early testicular differentiation in medaka. Gene Expr. Patterns 10, 283–289 (2010).
19. Paul-Prasanth, B., Shibata, Y., Horiguchi, R. & Nagahama, Y. Exposure to diethylstilbestrol during embryonic and larval stages of medaka fish (Orzias
latipes) lead to sex reversal in genetic males and reduced gonad weight in genetic females. Endo- roph. 152, 707–717 (2011).

20. Nakamura, M., Hourigian, T. F., Yamauuchi, K., Nagahama, Y. & Grau, E. G. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse Thalassoma duperrey. Environ. Biol. Fishes 24, 117–136 (1989).

21. Ijiri, S. et al. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia Oreochromis niloticus. Biol. Reprod. 78, 333–341 (2008).

22. Wang, X. G. & Orban, L. Anti-mullerian hormone and 11β-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev. Dyn. 236, 1329–1338 (2007).

23. Zou, K. et al. Identification of germline stem cells in the ovary of the teleost medaka. Science 328, 1561–1563 (2010).

24. Johnson, J., Canning, J., Kaneko, T., Pru, J. K. & Tilly, J. L. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428, 145–150 (2004).

25. Matson, C. K. & Zarkower, D. Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. Nat. Rev. Genet. 13, 163–174 (2012).

26. Nakamura, M., Bhandari, R. K. & Higa, M. The role of estrogens play in sex differentiation and sex changes of fish. Fish Physiol. Biochem. 28, 113–117 (2003).

27. Kawahara, T. & Yamashita, T. Anti-mullerian hormone of teleosts. Fish Physiol. Biochem. 28, 293–298 (2003).

28. Nakamura, M., Hourigan, T. F., Yamauchi, K., Nagahama, Y. & Grau, E. G. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse Thalassoma duperrey. Environ. Biol. Fishes 24, 117–136 (1989).

29. Wang, X. G. & Orban, L. Anti-mullerian hormone and 11β-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev. Dyn. 236, 1329–1338 (2007).

30. Zou, K. et al. Production of offspring from a germline stem cell line derived from neonatal oocytes. Nat. Cell Biol. 11, 631–636 (2009).

31. Matson, C. K. & Zarkower, D. Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. Nat. Rev. Genet. 13, 163–174 (2012).

32. Nakamura, M., Bhandari, R. K. & Higa, M. The role of estrogens play in sex differentiation and sex changes of fish. Fish Physiol. Biochem. 28, 113–117 (2003).

33. Kawahara, T. & Yamashita, T. Anti-mullerian hormone of teleosts. Fish Physiol. Biochem. 28, 293–298 (2003).

34. Nakamoto, M. et al. Cloning and expression of medaka cholesterol side chain cleavage cytochrome P450 during gonadal development. Dev. Growth Differ. 52, 385–395 (2010).

35. Rahman, M. S., Takemura, A. & Takano, K. Lunar synchronization of testicular development in rabbitfish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 367–373 (2001).

36. Nash, J. P. et al. An enzyme linked immunosorbant assay (ELISA) for testosterone, estradiol, and 17,20f-hydroxy-4-pregnen-3-one using acetycholinesterase as tracer: application to measurement of diet patterns in rainbow trout (Oncorhynhys mykiss). Fish Physiol. Biochem. 22, 355–363 (2000).

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Author contributions

B.P.-P. and R.K.B. are co-first authors. B.P.-P. was responsible for the design, competition and analysis of aromatase inhibition and sex-reversal of medaka and co-wrote the manuscript. R.K.B. carried out the sex-reversal experiments in tilapia and co-wrote the manuscript. T.K. performed dmrt1 expression in tilapia and analyzed the results of sex-reversal in tilapia. R.H. helped in aromatase inhibitor treatment of medaka and performed in situ hybridization of medaka gonads. Y.K. performed the hormone assays. M.N. performed two-color in situ hybridization of the medaka gonads. Y.S. performed the histological analysis of the gonads (control) of medaka, carried out artificial insemination in medaka along with B.P.-P. and R.H. and was responsible for the care of the experimental animal room for medaka. F.S. performed histological analysis of the gonads (control) of tilapia and was responsible for the care of the experimental animal room for tilapia. The co-corresponding authors M.N. and Y.N. conceived the experimental idea, developed the hypothesis, provided experimental design input, data interpretation and co-wrote the manuscript.

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ERRATUM: Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists

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In the original version of this Article the author M. Nakamura was incorrectly listed as having a current address of “National Institute for Basic Biology, Okazaki 444-8585, Japan”. This is the current address for the author M. Nakamoto. This has been corrected in the HTML and PDF versions of this Article.

In addition, the Supplementary Movies that accompany this study were omitted from the original version of this Article.