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

## SUBJECT AREAS:

OOGENESIS

STEROID HORMONES

DIFFERENTIATION

CELL LINEAGE

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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-17 $\beta$  (E<sub>2</sub>) 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 E<sub>2</sub> and androgen levels, secondary sexual characteristics, and male-like sex behavior, producing fertile sperm. Conversely, co-treatment of E<sub>2</sub> 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 E<sub>2</sub> 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 differentiated<sup>1</sup>. 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 paper<sup>2</sup> 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 granulosa cells<sup>3</sup> and the DMRT1 transcription factor in adult Sertoli cells<sup>4</sup> could reprogram granulosa cells into Sertoli cells and Sertoli cells into granulosa cells, respectively, showing that somatic cells in the gonads of male and female mice retained sexual plasticity even after terminal differentiation.

Among the vertebrates, teleost fishes display the greatest diversity of sexual phenotypes, thus providing an excellent model to investigate molecular mechanisms of sex determination/differentiation and sexual plasticity<sup>1</sup>. In the present study, we used two gonochoristic fish species, Nile tilapia, *Oreochromis niloticus*, and Japanese medaka, *Oryzias latipes*, for the demonstration of sexual plasticity in adult vertebrates. Like mammals, these species do not change sexual phenotypes after the gonads become terminally differentiated and mature. Both in tilapia and medaka, sex is determined chromosomally, but the chromosomes are homomorphic. Sex-determining gene of tilapia is yet to be discovered, but medaka is the second vertebrate from which a male sex-determining gene, *dmy/dmrt1bY*<sup>5,6</sup>, was identified, after the discovery of the mammalian sex-determining gene *SRY/Sry*<sup>7,8</sup>.

Accumulating evidence indicates that estrogens, principally estradiol-17 $\beta$  (E<sub>2</sub>) play a critical role in the differentiation and maintenance of an ovarian phenotype in a variety of vertebrates<sup>1,9</sup>. Based on the assumption that ovarian differentiation in tilapia and medaka must be both induced and constantly maintained by estrogens, we attempted to change the sex of adult females (XX) by removing estrogens through *in vivo* inhibition of aromatase, the terminal enzyme responsible for E<sub>2</sub> production, with highly specific aromatase inhibitors (AI). We used



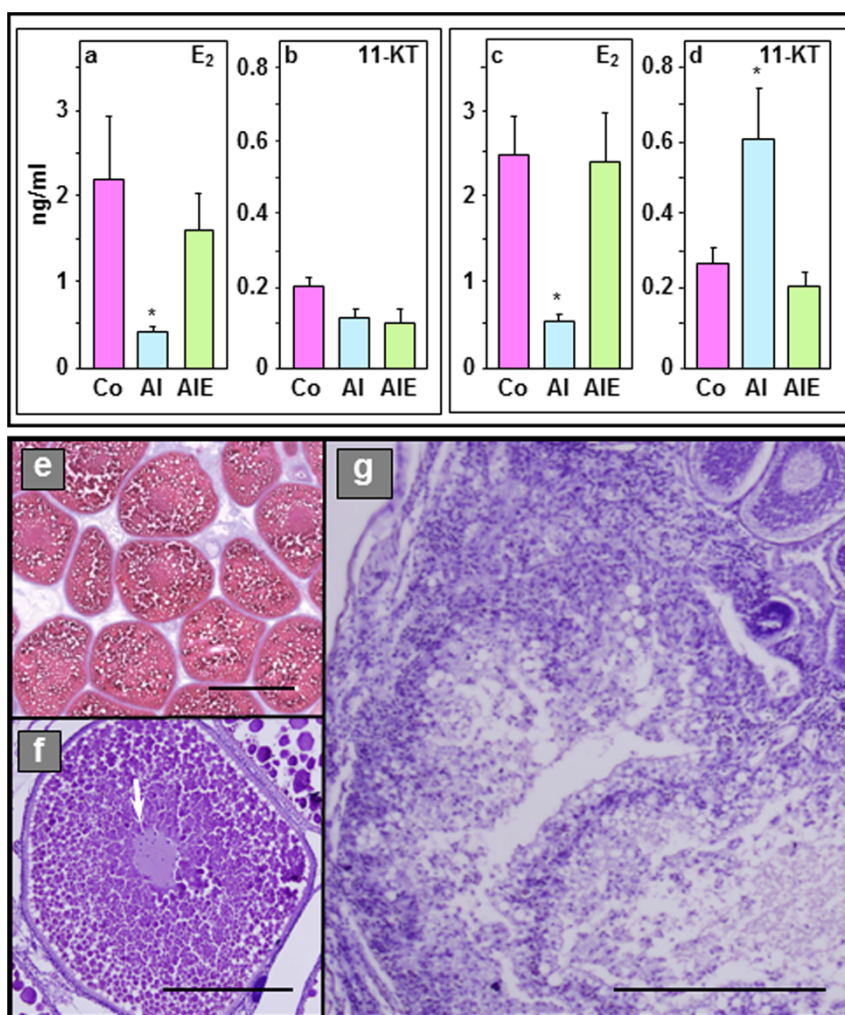
fadrozole (Fd) for tilapia and exemestane (EM) for medaka. The efficacy of Fd and EM in the induction of testicular differentiation in embryos of several non-mammalian vertebrates has already been proven<sup>10,11</sup>. Adult breeding females of tilapia (one year old) and medaka (5 months old) were 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

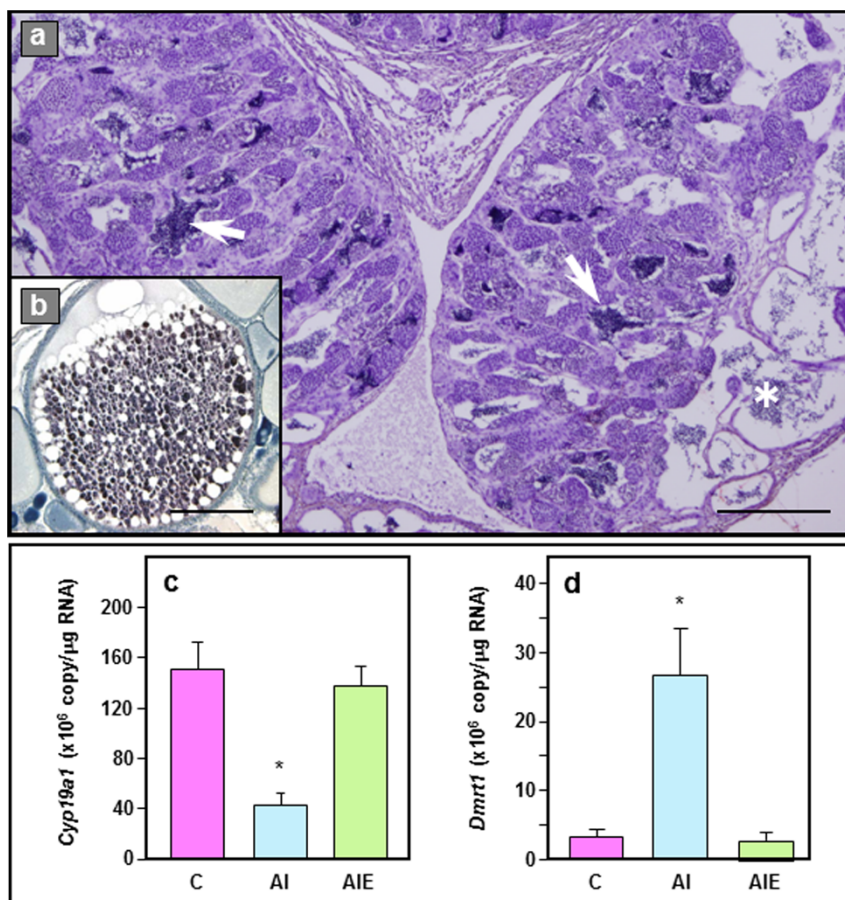
**E<sub>2</sub> depletion induces male-specific gonadal phenotype in adult genetic female tilapia.** In all adult breeding females of tilapia treated with AI alone, plasma levels of E<sub>2</sub> were significantly lower than those of the control groups (Fig. 1a and c). In contrast, no discernible changes 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 E<sub>2</sub> and 11-KT levels in fish

with co-treatment of E<sub>2</sub> 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 E<sub>2</sub> had ovaries with follicles at different stages of development comparable to those in the control ovaries (Fig. 2b).



**Figure 1 | Effects of aromatase inhibition on synthesis of sex steroids and ovarian morphology.** Plasma levels of E<sub>2</sub> (a) and 11-KT (b) in AI-treated XX tilapia at 90 dot and E<sub>2</sub> (c) and 11-KT (d) at 180 dot. Control XX tilapia (Co), AI-treated XX tilapia (AI), AI/E<sub>2</sub> co-treated XX tilapia (AIE). \* P < 0.05. (e) Ovarian morphology of untreated control XX tilapia at 90 dot. (f) A post-vitellogenic follicle in the ovary of an untreated control XX tilapia at 90 dot. (g) Ovarian morphology of AI-treated XX tilapia at 90 dot. Scale bars are 2 mm (e), 1 mm (f) and 100 µm (g).



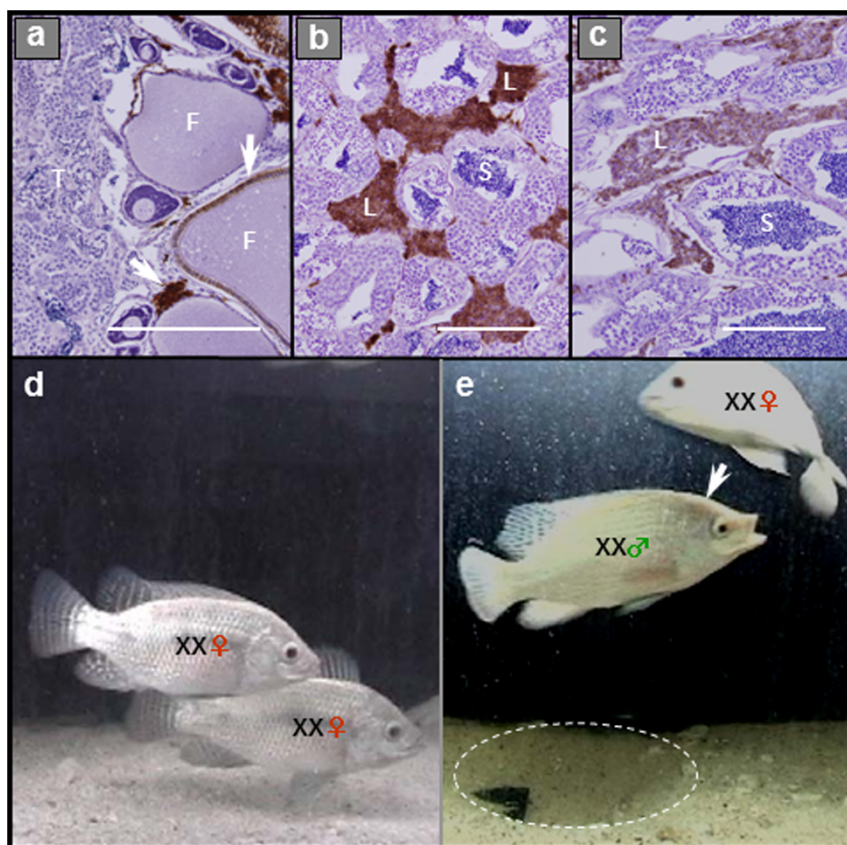
**Figure 2 | Gonadal morphology and expression of granulosa and Sertoli cell-specific markers after 180 days of AI treatment.** (a) Ovary of an AI-treated XX tilapia having fully transformed into a testis with spermatozoa and sperms (white arrow). Newly formed sperm ducts (white star). (b) An intact mature follicle in the ovary of an AI/E<sub>2</sub>-treated XX tilapia. Scale bars are 100 μm (a) and 1 mm (b). (c) Relative expression levels of the granulosa cell-specific marker, *cyp19a1*, in the ovaries of AI-treated XX tilapia at 180 dot. (d) Relative expression levels of the Sertoli cell-specific marker, *dmrt1*, in the ovaries of AI-treated XX tilapia at 180 dot. (c) and (d) Control XX tilapia (C), AI-treated XX tilapia (AI), AI/E<sub>2</sub> co-treated XX tilapia (AIE). \* P < 0.05.

**E<sub>2</sub> 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-form<sup>12–14</sup>. 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 production<sup>13,14</sup>. 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 E<sub>2</sub> 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 part 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 Δ<sup>5</sup>-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 tilapia<sup>15</sup>. 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 E<sub>2</sub> (Fig. 2d).

**E<sub>2</sub> 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-reversed 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.

**E<sub>2</sub> 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





**Figure 3 | Effects of AI on Leydig cell differentiation and secondary sex characteristics of XX tilapia.** (a) Cyp19a1 expression (white arrows) in the remaining oocytes and interstitial cells of sex-reversed XX tilapia at 180 dot. Follicle (F), Testis (T). (b) Expression of Cyp11a1 in the *de novo* differentiated Leydig cells (L) interspersed between the spermatogenic tissue (S) in the newly-formed testis of sex-reversed XX tilapia at 180 dot. (c) Expression of  $3\beta$ -HSD in the Leydig cells (L) interspersed between the spermatogenic tissue (S) of sex-reversed XX tilapia at 180 dot. Scale bars are 100  $\mu$ m. (d) Secondary sex characteristics of untreated control XX tilapia. (e) Secondary sex characteristics and sex behavior of an AI-treated XX tilapia (180 dot) paired with a control XX tilapia. Sex-reversed XX tilapia with pink coloration at the extremities of the head (arrow) makes nest (dotted circle) in the sand at the bottom of the fish tank. The authors thank Dr. Yasuhisa Kobayashi for capturing the photographs shown in Fig. 3 (d) and (e).

unaltered by hormone treatments<sup>15,16</sup>. 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  $E_2$  in the ovaries of the exposed fish. Control fish treated with the vehicle (99% ethanol) showed more amount of  $E_2$  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).

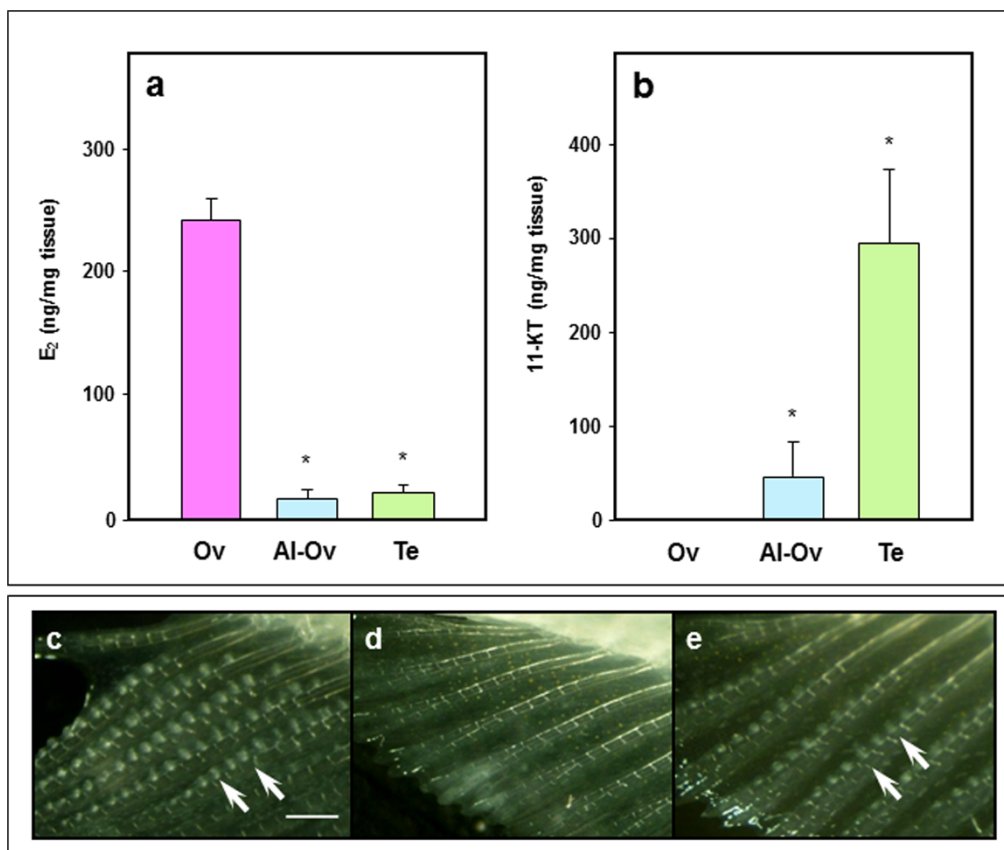
**$E_2$  depletion induces male-specific gonadal phenotype in adult, genetic female medaka.** The ovaries of medaka prior to AI treatment and control fish contained numerous pre-vitellogenic and vitellogenic follicles together with some post-ovulatory follicles indicating that the fish were ovulating 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 *dmy* and *dmrt1* (Data not shown). Their genomes were free of *dmy*. They exhibited normal development and fertility, producing progeny in normal sex ratios like any other normal females (Fig. 6e).

**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





**Figure 4 | Effects of AI treatment on synthesis of sex steroids and secondary sex characteristics in XX medaka.** (a) Tissue levels of E<sub>2</sub> in the ovary of AI-treated XX medaka. (b) Tissue levels of 11-KT in the ovary of AI-treated medaka. (a) and (b) Control Ovary (Ov), ovary of AI-medaka (AI-Ov), Control testis (Te), \* P < 0.05 (Student's *t*-test). (c) Papillar processes (white arrows) in the anal fin of XY male medaka. Scale bar is 80  $\mu$ m. (d) Anal fin of XX female medaka. No papillar processes are seen. (e) Emergence of papillar processes (white arrows) after 10 days of AI treatment in the anal fin of XX female medaka. Scale bar for (d) and (e) are the same as in (c).

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 cells<sup>17</sup>. More recently, we showed that a new factor, *gonadal-soma derived factor* (*gsdf*), is also expressed in Sertoli cells and is associated with testicular differentiation<sup>18</sup>. Unlike *dmy*, the expression of *gsdf* is closely linked to the gonadal phenotype<sup>18,19</sup>. 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 germline stem cells (GSCs) in the germinal 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* (*olvas* 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).

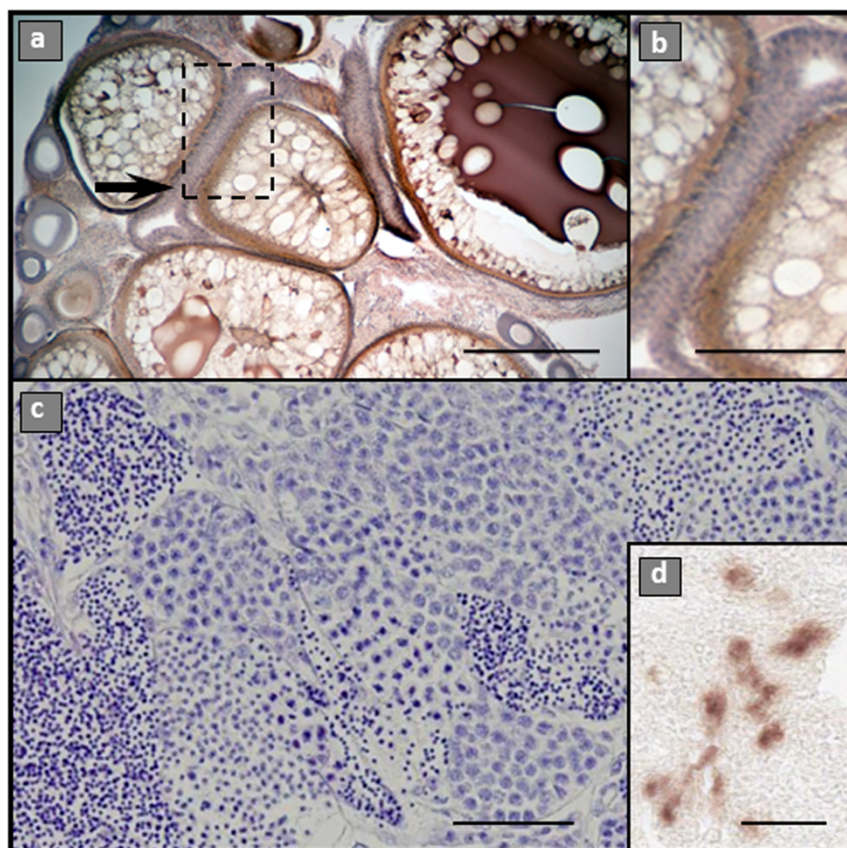
In medaka, gonads at 30 dot contained not only spermatogonial cysts, but also atretic follicles (Fig. 8g). Although *foxl2* was expressed in these atretic follicles, neither granulosa cell (*cyp19a1*) nor Sertoli

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 *Foxl2*<sup>3</sup> and *Dmrt1*<sup>4</sup> 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.

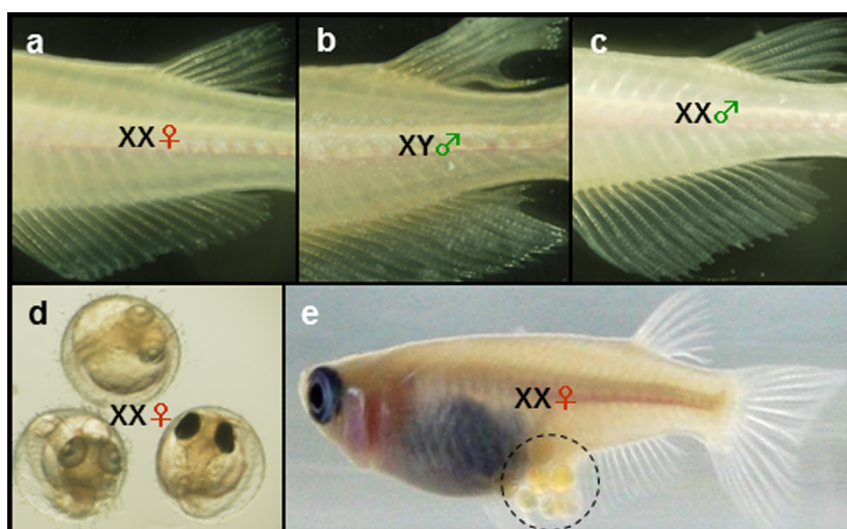
The first obvious signs of morphological changes in XX gonads of tilapia and medaka seen after the onset of AI treatment were the



**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  $\mu\text{m}$  (a), 250  $\mu\text{m}$  (b), 80  $\mu\text{m}$  (c) and 50  $\mu\text{m}$  (d).

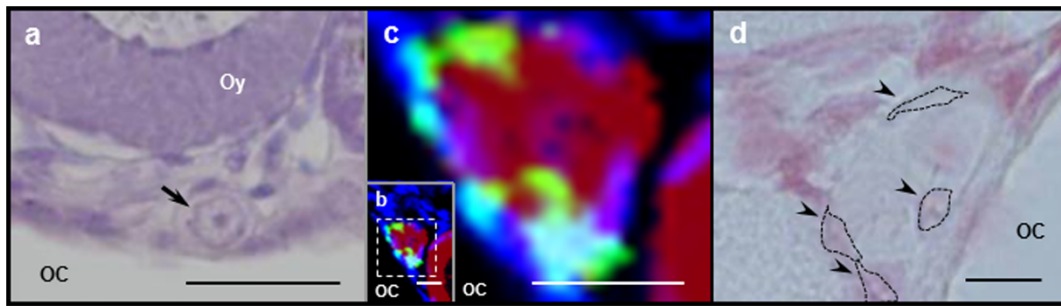
degeneration of female germ cells. The specific action of AIs on these morphological changes was confirmed by another AI group of tilapia co-treated with  $E_2$ . Plasma  $E_2$  levels of these fish were comparable to those of controls. Co-treatment of  $E_2$  could rescue the germ cells of

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

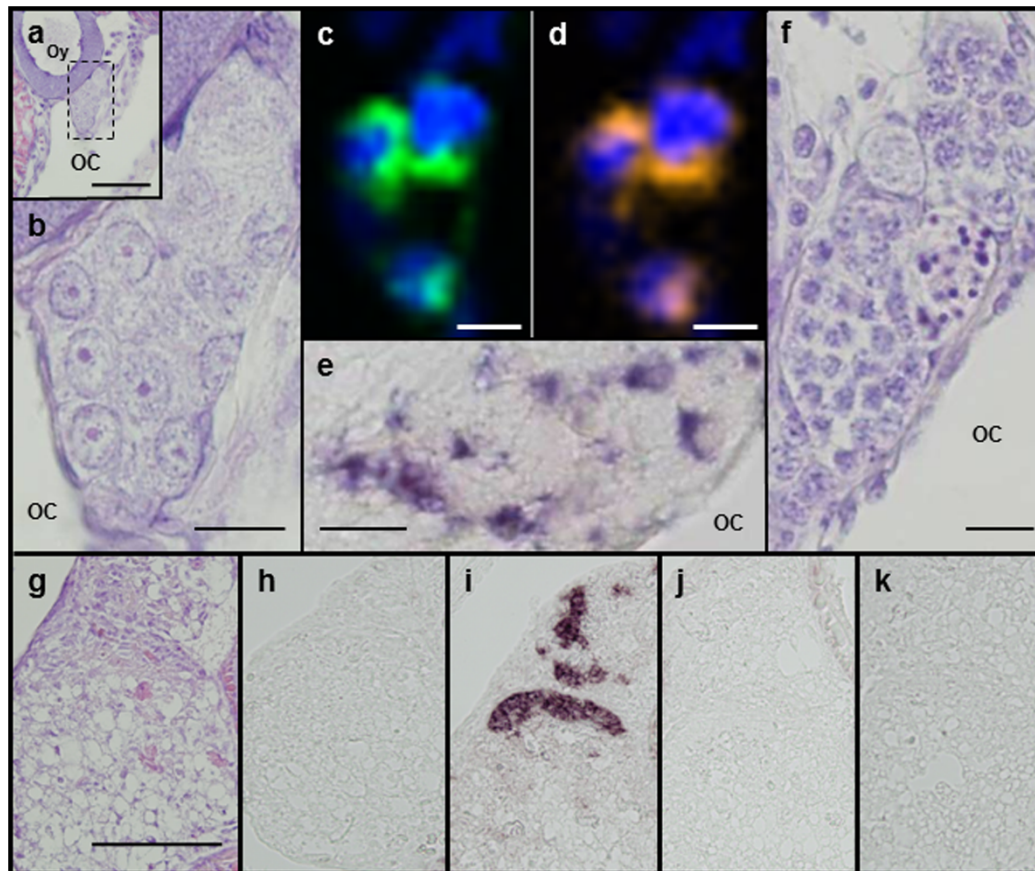


**Figure 6 | Fin structures and fertility assessment of sex-reversed XX medaka.** (a) Fin morphology of the control XX female medaka at 60 dot. Small anal and unforked dorsal fins are shown. (b) Fin morphology of an XY male medaka. Anal fin is large and dorsal fin is forked. (c) Fin morphology of a sex-reversed 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 *gsdf*. No expression was detectable. The section is counter-stained with hematoxylin-eosin. Scale bars are 80  $\mu\text{m}$  (a), 50  $\mu\text{m}$  (b), 50  $\mu\text{m}$  (c) and 5  $\mu\text{m}$  (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 *gsdf* (orange) at 30 dot. Images shown in (c) and (d) are of the same section hybridized with amplified RNAs of *sox9b* and *gsdf*. Nuclei are stained with DAPI (blue). (e) *gsdf* expression in the somatic cells around the spermatogenic tissue of sex-reversed XX medaka at 50 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 *cyp19a1a* at 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 *gsdf* 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  $\mu\text{m}$  (a), (c), (d), 10  $\mu\text{m}$  (b), (e), (f) and





protogynous wrasse, *Thalassoma duperrey*<sup>20</sup>. In wrasse, the initial stage of sex reversal was accompanied by a decrease in plasma levels of E<sub>2</sub>, proving that E<sub>2</sub> 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*<sup>3</sup>. Ovaries of these mice had a drop in serum E<sub>2</sub> 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 3 $\beta$ -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 *cyp11b1* in XY tilapia<sup>21</sup>. The study in the zebrafish, *Danio rerio*<sup>22</sup> also found that the expression of 11 $\beta$ -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 E<sub>2</sub> 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 E<sub>2</sub> 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 E<sub>2</sub>, 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<sup>23</sup>. 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<sup>24,25</sup>, 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<sup>8</sup>. When the XY embryos were treated with E<sub>2</sub><sup>18</sup> or diethylstilbestrol<sup>19</sup>, 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<sup>3</sup> 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<sup>4</sup>. 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 E<sub>2</sub> 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<sup>3</sup> and DMRT1<sup>4,26</sup> 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 germline stem cell biology in vertebrates.



## Methods

**Animals.** Tilapia. Cichlid fish (Nile tilapia, *Oreochromis niloticus*) were used as the experimental animals. An 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 aquariums, where they were reared until experimentation under natural photoperiod. The fish were fed with commercial salmon feed (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 Qurt 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 26–28°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 was refrigerated until further use. For the preparation of AI and E<sub>2</sub> containing feed, E<sub>2</sub> 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 the control feed and the other with AI-feed at 200 µg/g concentration. The effective dose was selected from a previous experiment published elsewhere<sup>27</sup>. The third group was fed with AI and E<sub>2</sub> (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 AI group.

**Medaka.** There have been reports that treatment with fadrozole during early development failed to induce complete sex-reversal in medaka<sup>28,29</sup>. Therefore, we chose the steroidal inhibitor of aromatase, EM (Wako Chemicals, Tokyo, Japan) to inhibit E<sub>2</sub> production in medaka. Each 3-L fish tank (Nissoh, Japan) contained 5 adult breeding females and a single adult male. Fertility of the males also was tested prior to the experiments. Stock solution of EM was prepared in 99.9% ethanol (Wako Chemicals) and was used directly for the treatments. The drug was added to the water in the experimental tanks. Initially, two doses, 10 and 100 µg/L, were administered to determine the effective doses required for complete sex reversal. An equal volume of ethanol was added to the water in control tanks. Water in both control and experimental tanks was changed every 24 h and the drug was freshly added. Sex reversal was considered to be complete if the histological observation of the gonads showed a testis instead of an ovary. Since complete sex reversal was observed only in fish exposed to 100 µg/L, we used only this concentration for further studies. In subsequent experiments, sampling was done every 10 days in order to analyze the process of sex change. Changes in secondary sexual characteristics were noted periodically. All the fish were fed with artemia 3 times a day.

**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.<sup>30</sup>.

**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 ISH. Samples for histology, ISH and IHC 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*, *foxl2*, *gsdf* and *sox9b*. Hybridization signals were detected using alkaline phosphatase 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 *sox9b* and *olvas* following previously described methods<sup>31</sup> (S5). *sox9b* probe was labeled with DIG and *olvas* 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 observed and photographed using a confocal microscope (Zeiss 710, Carl-Zeiss, Germany). Colors were assigned digitally to the hybridization signals of *sox9b* (green), *olvas* (red) and *gsdf* (orange) using Zeiss software provided by the manufacturer (Carl-Zeiss).

**Hormone measurement.** Tilapia. Plasma levels of E<sub>2</sub> and 11-KT of tilapia were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described by Rahman et al.<sup>32</sup>. 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 E<sub>2</sub> and 11-KT in gonadal tissue were measured by ELISA according to the descriptions in Nash et al.<sup>33</sup>. 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 E<sub>2</sub> (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 by using 0.1 pmoles of Oligo (dT)<sub>20</sub> 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) by using Student's *t*-test at 95% level of significance (SPSS 9.0). To compare significant differences between control, AI-treated and AI/E<sub>2</sub>-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 between untreated female and treated female and untreated female and male using Student's *t*-test (Graphpad Prism) at 95% level of significance.

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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, experimentation 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 dmr1 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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## Additional information

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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.

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