Article

Steroid hormone-deprived sex reversal in *cyp11a1* mutant XX tilapia experiences an ovary-like stage at molecular level

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Fish sex is largely influenced by steroid hormones, especially sex hormones. Here, we established a steroid hormone-free genetic model by mutation of *cyp11a1* in Nile tilapia, which was confirmed by EIA assay. Gonadal phenotype and transcriptome analyses showed that the XX mutants displayed sex reversal from female to male but with defective spermatogenesis. Despite the sex reversal, the aromatase encoding gene *cyp19a1a* was continuously expressed in the gonads of the XX mutants, which might be caused by androgen deficiency. Whole-mount fluorescence in situ hybridization and transcriptome analysis showed that the gonads of the XX mutants firstly developed towards ovary but shifted to testis between 10 to 15 days after hatching. Detailed expression analysis of key sex differentiation pathway genes *foxl3* and *dmrt1* combined with apoptosis analysis revealed transdifferentiation of germ cells from female to male during sex reversal. Rescue experiments showed that both P5 and E2 treatment rescued the sex reversal of *cyp11a1* mutant XX fish. Overall, our results revealed a transient ovary-like stage and transdifferentiation of germ cells from female to male in the early gonads of the steroid hormone-deprived *cyp11a1* mutant XX fish.

Steroid hormones, including glucocorticoids, mineralocorticoids, estrogens, androgens, and progestins, are important endocrine regulators in vertebrates[1]. *cyp11a1* encodes cholesterol side-chain cleavage enzyme, the only enzyme currently known to catalyze the conversion of cholesterol to pregnenolone (P5), the precursor of all steroid hormones[2]. Case reports in humans show that mutation in *CYP11A1* is lethal due to severe adrenal insufficiency[3–6]. The lethality is also observed in rabbits and mouse after *Cyp11a1* mutation[7,8]. These studies in mammals have provided solid evidence for the critical role of *Cyp11a1* in steroid hormone production and homeostasis maintenance. Different from the lethality of *Cyp11a1* mutation reported in mammals, recent studies have shown that zebrafish *cyp11a2* (the counterpart of *cyp11a1* in vertebrates) mutants survive to adult[9,10]. However, unlike other fish species and tetrapods, zebrafish has two *cyp11a* genes (*cyp11a1* and *cyp11a2*), and both of them are expressed in steroidogenic tissues[11]. Compensation of *cyp11a1* may exist when *cyp11a2* is mutated. Therefore, it remains unknown whether *cyp11a1* mutants are viable in other fish species.

Fish sex is largely influenced by steroid hormones, especially sex hormones. The essential role of estrogen in fish ovary development has been widely accepted. Blocking estrogen synthesis has been shown to result in the transition of undifferentiated ovary to testis in many fish species[12–22], even differentiated ovary to testis in medaka and tilapia[23,24]. However, the dependence of early ovary differentiation on estrogen seems to vary among different fish species. In medaka, the expression of the aromatase encoding gene *cyp19a1a* is first detected in the ovary from 4 to 10 days after hatching (dah), after initiation of oogenesis[25,26]. Blocking estrogen synthesis through aromatase inhibitor (AI) treatment or *cyp19a1a* mutation does not affect the early oogenesis and folliculogenesis in female medaka[21,26]. Zebrafish is considered a juvenile hermaphroditic fish, as the gonads develop as juvenile ovary first in both females and males[27]. Mutation of *cyp19a1a*, *cyp17a1*, or *cyp11a2* leads to estrogen deficiency and all-male development but does not affect the formation of juvenile ovary and oocyte-like germ cells at early developmental stages in zebrafish[10,18,22]. These results indicate that estrogen is not necessary for early ovary differentiation in medaka and zebrafish. In Nile tilapia, *cyp19a1a* expression is detected in the gonads of XX fish at 5 dah, the critical time for tilapia molecular sex differentiation[28,29]. Blocking estrogen synthesis in XX fish by *cyp19a1a* or *cyp17a1* mutation has been reported to result in female-to-male sex reversal[20,30], whether sex reversal caused by estrogen deficiency in tilapia experiences an ovary stage like medaka and zebrafish or experiences an ovary-like stage in gene expression remains to be investigated.

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The *cyp11a1*-free genetic model lacks all steroid hormones. When combining this model with hormone treatment, it is possible to study the physiological effects of a certain hormone. To date, this genetic model has not been established in fish with only one *cyp11a* gene. Nile tilapia (*Oreochromis niloticus*) is a gonochoristic fish with an XX/XY sex-determining system. The availability of sex-linked genetic marker[31], well-established gene editing technique[32], and medium body size which facilitates blood collection and hormone analysis, make tilapia a good model for gene function analysis and endocrine research. In this study, we established a viable steroid hormone-free genetic model in Nile tilapia by mutation of *cyp11a1*, investigated the role of sex steroid hormone in fish gonad differentiation by analyzing the gonad development of the mutants, combined with rescue experiments and luciferase assay.

Results

Establishment of steroid hormone-free genetic model by mutation of *cyp11a1* in tilapia

In tilapia, *cyp11a1* was mainly expressed in the liver, ovary, testis, and head kidney as revealed by qPCR (Supplementary Fig. 1A). Gonadal transcriptome analysis showed that *cyp11a1* displayed sexual dimorphic expression during development, with higher expression in XX gonads at 5, 7, 20, 30, and 40 dah and higher expression in XY gonads at 90 and 180 dah (Supplementary Fig. 1B). In situ hybridization results showed that *cyp11a1* was expressed in ovarian theca cells, testicular Leydig cells and head kidney interrenal cells (Supplementary Fig. 1C–K). To get a better understanding of the physiological role of steroid hormones, we established a steroid hormones-free genetic model in tilapia by mutation of *cyp11a1* using CRISPR/Cas9 (Fig. 1A, B). Homozygous mutant (*cyp11a1*$^{-/-}$) fish with 22 bp deletions were successfully identified by *Sau*96I digestion and PAGE (Fig. 1C). The deletion was further verified by Sanger sequencing (Fig. 1D). This deletion resulted in a truncated protein lacking adrenodoxin binding domain and heme binding domain due to the premature termination of

cyp11a1 translation (Supplementary Fig. 2A). EIA results showed that the *cyp11a1*$^{-/-}$ fish displayed background levels of serum P5, cortisol, E2, DHP, T and 11-KT compared with wild-type (WT) fish (Fig. 1E–J). Even though the *cyp11a1*$^{-/-}$ fish displayed decreased survivability under hypoxic conditions (Supplementary Fig. 2B, C), the fish could survive under normal water conditions with enough oxygen. The survival of *cyp11a1*$^{-/-}$ fish makes it a feasible steroid hormone-free genetic model.

Mutation of *cyp11a1* results in female-to-male sex reversal of XX fish and defects in spermatogenesis

Histological analyses showed that the gonads of the WT XX fish and WT XY fish at 90 dah developed into ovaries and testes, respectively, while the gonads of both *cyp11a1*$^{-/-}$ XX and *cyp11a1*$^{-/-}$ XY fish developed into testes, indicating female to male sex reversal of XX fish after *cyp11a1* mutation (Fig. 2A–H). IF results showed that Cyp19a1a was expressed in the gonads of WT XX, but not in the gonads of WT XY and *cyp11a1*$^{-/-}$ XY fish. Unexpectedly, it was still expressed in the gonads of *cyp11a1*$^{-/-}$ XX fish. Cyp11c1 was expressed in the gonads of WT XY, *cyp11a1*$^{-/-}$ XX, and *cyp11a1*$^{-/-}$ XY fish, but not in the gonads of WT XX fish (Fig. 2I–P). To get a comprehensive understanding of gene expression in the gonads of *cyp11a1*$^{-/-}$ XX and *cyp11a1*$^{-/-}$ XY fish, transcriptome sequencing was performed. Pearson correlation analysis revealed that the global gene expression pattern in the gonads of *cyp11a1*$^{-/-}$ XX and *cyp11a1*$^{-/-}$ XY fish was highly similar to that in the gonads of WT XY fish but not WT XX fish (Fig. 2Q). Accordingly, more differentially expressed genes were detected in the gonads when comparing *cyp11a1*$^{-/-}$ XX or *cyp11a1*$^{-/-}$ XY fish to WT XX fish than comparing to WT XY fish ($|\log_2 FC| \geq 1$, $P < 0.01$) (Fig. 2R). Detailed gene expression analysis revealed downregulation of female pathway gene *foxl2*, and upregulation of male pathway gene *dmrt1* in the gonads of the *cyp11a1*$^{-/-}$ XX fish compared with WT XX fish (Fig. 2S). Consistent with the IF results, both *cyp19a1a* and *cyp11c1* were expressed in the gonads of the *cyp11a1*$^{-/-}$ XX fish (Fig. 2S). Further analysis revealed upregulation of steroidogenic factor *sf1*,
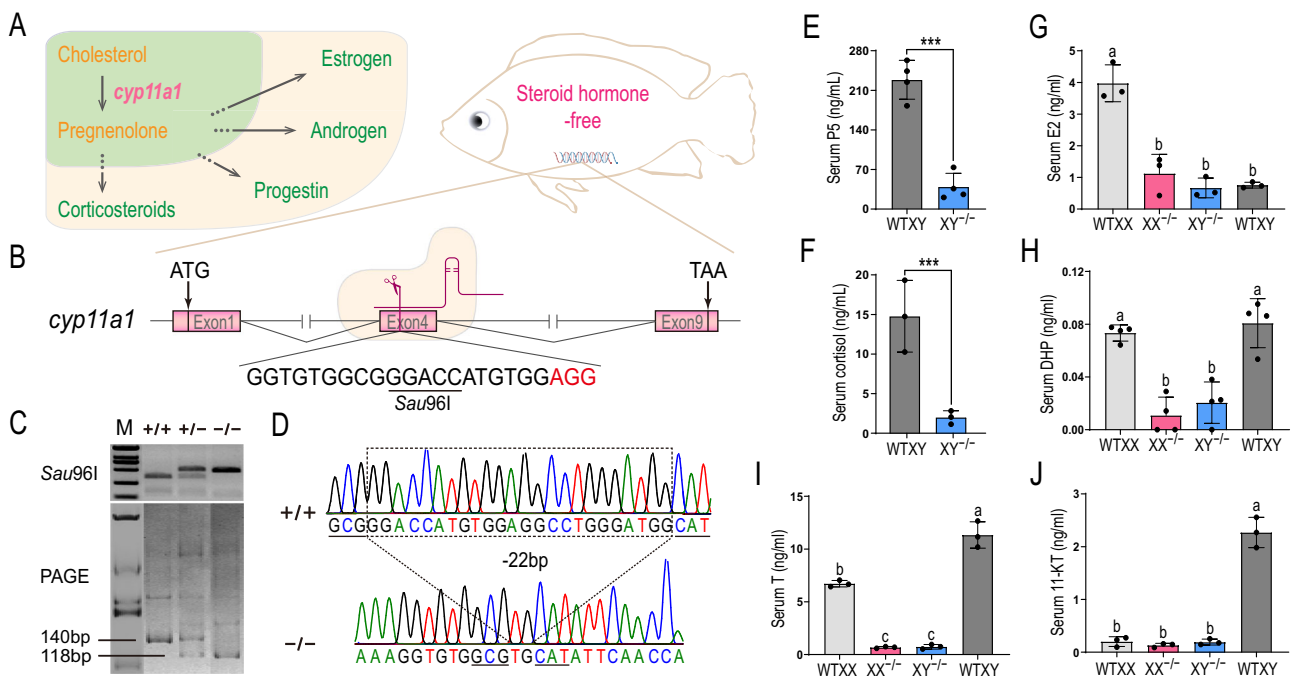

Fig. 1 | Establishment of steroid hormone-free genetic model by mutation of *cyp11a1* in tilapia. A Simplified diagram displaying the position of *cyp11a1* in the steroidogenic pathway. **B** Target site in *cyp11a1*. A target site with a *Sau*96I restriction enzyme site (underlined) was designated in the fourth exon of *cyp11a1*. The PAM sequence is marked in red. **C** Mutation identification by *Sau*96I digestion and PAGE. "+/+", "+/−", "−/−" indicate wild-type fish, heterozygous mutants, and homozygous mutants, respectively. **D** Verification of mutation by Sanger

sequencing. A 22 bp deletion was detected in *cyp11a1*$^{-/-}$ fish. **E–J** Serum P5, cortisol, E2, DHP, T, and 11-KT level of the WT and *cyp11a1*$^{-/-}$ fish. Values are presented as mean ± SD ($n \geq 3$/genotype). "***" above the error bar in (**E, F**) indicates statistically significant differences at $P < 0.001$ as determined by a two-tailed independent Student's *t*-test. Different letters above the error bar indicate statistically significant differences at $P < 0.05$ as determined by one-way ANOVA followed by the Tukey test for multiple comparisons.
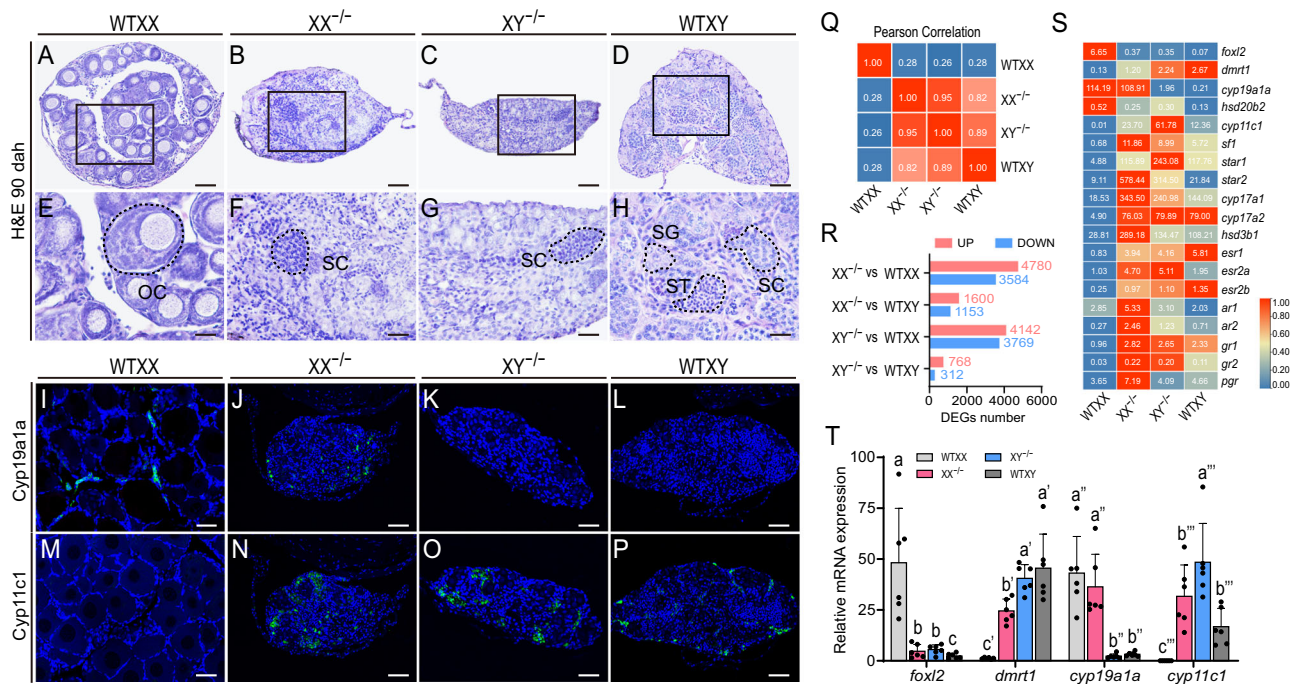
Fig. 2 | Female-to-male sex reversal of XX fish after *cyp11a1* mutation. A–H H&E staining of gonads from the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, *cyp11a1*⁻/⁻ XY fish at 90 dah. **E–H** is the higher magnification of the boxed area (black boxes) in (**A–D**). Black dotted lines outline oogonia (OG), oocytes (OC), spermatogonia (SG), spermatocytes (SC), and spermatids (ST) in the gonads. Scale bar: **A–D** 50 μm; **E–H** 20 μm. **I–P** Immunofluorescence for Cyp19a1a and Cyp11c1 in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, *cyp11a1*⁻/⁻ XY fish at 90 dah. The positive signal corresponds to green fluorescence. The nuclei were stained with DAPI (blue fluorescence). Scale bar, 50 μm. **Q** Pearson correlation analysis of gene expression in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and *cyp11a1*⁻/⁻ XY fish at 90 dah. The number inside the square is the Pearson correlation coefficient between two samples. The closer the correlation coefficient gets to 1, the higher the similarity of global gene expression patterns between samples. **R** The number of DEGs in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and *cyp11a1*⁻/⁻ XY fish at 90 dah.

Values on the right side of the column represent the number of up- or down-regulated genes. **S** The expression of genes related to sex differentiation (*foxl2* and *dmrt1*), steroid hormone synthesis (*cyp19a1a*, *hsd20b2*, *cyp11c1*, *sf1*, *star1*, *star2*, *cyp17a1*, *cyp17a2*, and *hsd3b1*) and response (*esr1*, *esr2a*, *esr2b*, *ar1*, *ar2*, *gr1*, *gr2*, and *pgr*) in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and *cyp11a1*⁻/⁻ XY fish at 90 dah. The number inside the square is the RPKM value of genes in each sample. The color from blue to red represents the expression of genes from low to high in each sample. **T** Relative mRNA expression level of *foxl2*, *dmrt1*, *cyp19a1a*, and *cyp11c1* in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and *cyp11a1*⁻/⁻ XY fish at 90 dah by qPCR ($n = 6$/genotype). Values are presented as mean ± SD. Different letters above the error bar indicate statistically significant differences at $P < 0.05$ as determined by one-way ANOVA followed by the Tukey test for multiple comparisons. dah days after hatching.

steroidogenic acute regulatory protein coding gene *star1*, *star2*, steroidogenic enzyme coding genes *cyp17a1*, *hsd3b1*, estrogen receptor gene *esr2a*, androgen receptor genes *ar1*, *ar2*, glucocorticoids receptor genes *gr1*, *gr2*, progestin receptor gene *pgr* in the gonads of *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish compared with WT XX and WT XY fish (Fig. 2S). The expression of *foxl2*, *dmrt1*, *cyp19a1a*, and *cyp11c1* were further confirmed by qPCR (Fig. 2T). Intriguingly, we found that androgen (11-KT) treatment eliminated the expression of Cyp19a1a in the gonads of the *cyp11a1*⁻/⁻ XX fish as revealed by IF (Fig. 3A–E). In luciferase assay, 11-KT treatment inhibited the *sf1*-activated *cyp19a1a* transcription via AR1 and AR2. Also, synergistic inhibitory effects were observed for tilapia AR2 and Dmrt1 on *cyp19a1a* transcription (Fig. 3F).

The *cyp11a1*⁻/⁻ fish show androgen deficiency raised the question that if the mutants could process spermatogenesis. IF results revealed significantly reduced Vasa-positive germ cells in the gonads of *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish compared to those in the gonads of WT XY fish at 90 dah (Fig. 4A–F). Consistently, gonad transcriptome analysis revealed downregulation of germ cell marker genes *vasa*, *dazl*, spermatogonia marker genes *nanos2*, *hells*, primary spermatocytes marker genes *sycp3*, *dmc1*, secondary spermatocytes marker genes *ccna1*, *aruka*, spermatids marker genes *tex36*, *spata18* and mitosis-related genes *cenpf*, *ccnb3*, *plk1*, *mis18bp1*, *kif20a*, *mastl* in the gonads of the *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish compared with WT XY fish at 90 dah (Fig. 4G). Histological analysis showed that only a few spermatocytes and spermatids exist in the gonads of *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish at 180 dah, indicating severe

defects in spermatogenesis (Fig. 4H–M). Notably, both *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish displayed undersized testes at 90 and 180 dah, as reflected by the significantly decreased cross-sectional area of testes and GSI compared with WT XY fish (Supplementary Fig. 3A–C). To check whether fertile sperm was produced in the mutants, Pap staining, mobility test, and IVF assay were performed. Pap staining and mobility tests showed that no tailed and motile sperm was produced in the gonads of *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish at 180 dah (Fig. 4N–S). IVF results showed that both *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish were infertile (Fig. 4T). These results showed that the gonads of steroid hormone-free tilapia developed into testes in both XX and XY fish with defective spermatogenesis.

Transient ovary-like stage during sex reversal of *cyp11a1*⁻/⁻ XX fish
To make the sex reversal process clear, we tracked the expression of the male pathway gene *gsdf*[33,34] in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, *cyp11a1*⁻/⁻ XY fish at 5, 10, 15, and 25 dah by FISH. The results showed that *gsdf* was highly expressed in the gonads of the *cyp11a1*⁻/⁻ XY and WT XY fish but not in the gonads of the WT XX fish at 5, 10, 15, and 25 dah. In the gonads of the *cyp11a1*⁻/⁻ XX fish, *gsdf* expression was not detected at 5 and 10 dah but gradually upregulated at 15 and 25 dah (Fig. 5A–P, Q). IF results showed that Cyp19a1a was continuously expressed in the gonads of the *cyp11a1*⁻/⁻ XX fish at 5, 10, 15, and 25 dah (Fig. 5A–P, R). These results implied that the gonad of the *cyp11a1*⁻/⁻ XX fish may first develop toward the ovary but shifted its direction toward the testis during 10 to 15 dah. To investigate whether female germ cells exist in the gonads of the *cyp11a1*⁻/⁻
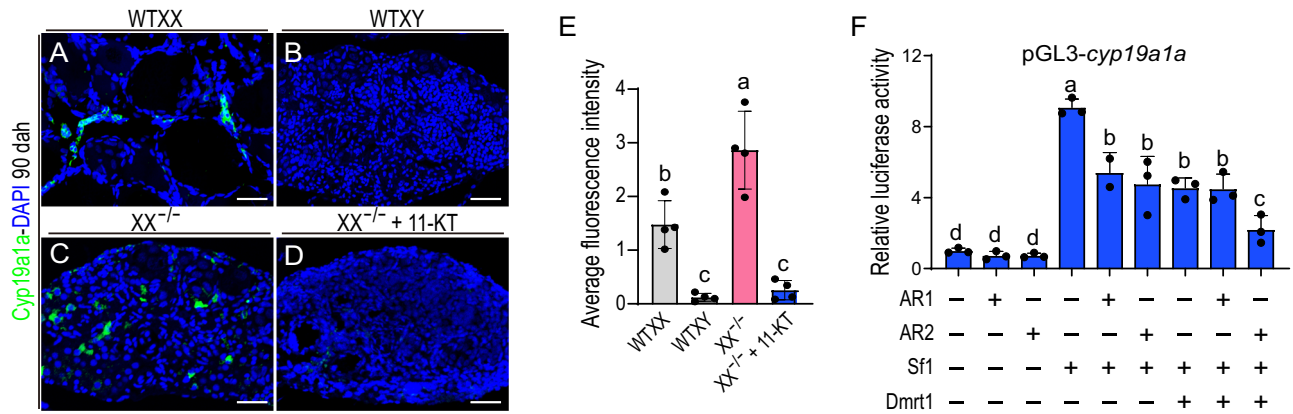
Fig. 3 | Downregulation of Cyp19a1a in gonads of *cyp11a1⁻/⁻* XX fish after 11-KT treatment. A–D Immunofluorescence for Cyp19a1a in the gonads of WT XX, WT XY, *cyp11a1⁻/⁻* XX, and 11-KT treated *cyp11a1⁻/⁻* XX fish at 90 dah. The positive signal corresponds to green fluorescence. The nuclei were stained with DAPI (blue fluorescence). Scale bar, 50 μm. **E** Quantification of average fluorescence intensity of Cyp19a1a positive signal in the gonads of the WT XX, WT XY, *cyp11a1⁻/⁻* XX, and 11-KT treated *cyp11a1⁻/⁻* XX fish at 90 dah (*n* = 4/genotype). **F** Effects of AR1, AR2, Sf1, Dmrt1 on the promoter activity of tilapia *cyp19a1a* (*n* = 3, technical replicates). Values are presented as mean ± SD. Different letters above the error bar indicate statistically significant differences at *P* < 0.05 as determined by one-way ANOVA followed by the Tukey test for multiple comparisons. dah days after hatching.
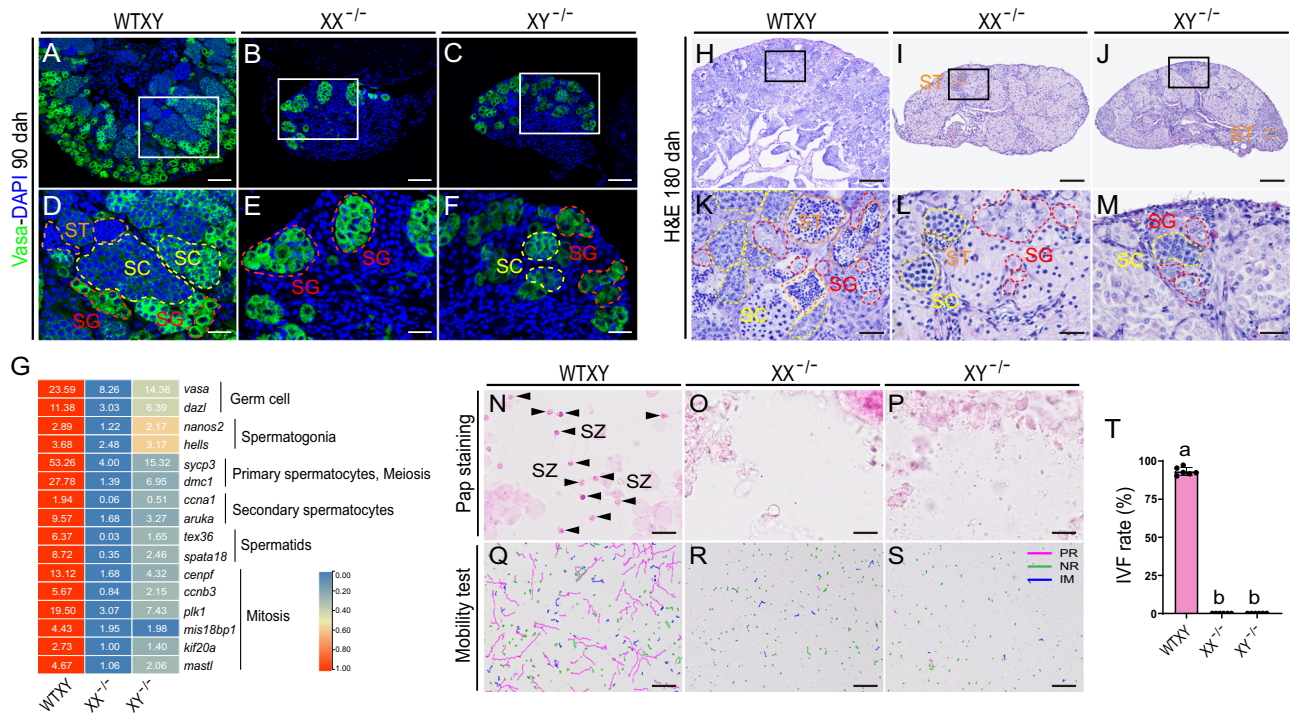

Fig. 4 | Defective spermatogenesis in gonads of *cyp11a1⁻/⁻* XX and XY fish.
A–F Immunofluorescence for Vasa in the gonads of the WT XY, *cyp11a1⁻/⁻* XX, and *cyp11a1⁻/⁻* XY fish at 90 dah. **D–F** is the higher magnification of the boxed area (white boxes) in (**A–C**). Red, yellow, and brown dotted lines outline spermatogonia (SG), spermatocytes (SC), and spermatids (ST), respectively. The positive signal corresponds to green fluorescence. The nuclei were stained with DAPI (blue fluorescence). Scale bar: **A–C** 50 μm; **D–F** 20 μm. **G** The expression of germ cell marker genes *vasa*, *dazl*, spermatogonia marker genes *nanos2*, *hells*, primary spermatocyte marker genes *sycp3*, *dmc1*, secondary spermatocyte marker genes *ccna1*, *aruka*, spermatid marker genes *tex36*, *spata18*, and mitosis-related genes *cenpf*, *ccnb3*, *plk1*, *mis18bp1*, *kif20a*, *mastl* in the gonads of the WT XY, *cyp11a1⁻/⁻* XX, and *cyp11a1⁻/⁻* XY fish at 90 dah. The number inside the square is the RPKM value of genes in each sample. The color from blue to red represents the expression of genes from low to high in each sample. **H–M** H&E staining of gonads from the WT XY,

cyp11a1⁻/⁻ XX, and *cyp11a1⁻/⁻* XY fish at 180 dah. **K–M** is the higher magnification of the boxed area (black boxes) in (**H–J**). Red, yellow, and brown dotted lines outline spermatogonia (SG), spermatocytes (SC), and spermatids (ST), respectively. Scale bar: **H–J** 100 μm; **K–M** 20 μm. **N–P** Papanicolaou (Pap) staining of testis mash in WT XY, *cyp11a1⁻/⁻* XX, *cyp11a1⁻/⁻* XY fish at 180 dah. Black arrowheads indicate spermatozoa. Scale bar, 10 μm. **Q–S** Detection of motile sperm in testis homogenate of WT XY, *cyp11a1⁻/⁻* XX, *cyp11a1⁻/⁻* XY fish at 180 dah. The red, green, and blue curves are the trajectories of progressive (PR), non-progressive (NR), and inmotile (IM) cells, respectively. The progressive cells in the testis were usually motile sperm. Scale bar, 50 μm. **T** Fertility assessment of WT XY, *cyp11a1⁻/⁻* XX, and *cyp11a1⁻/⁻* XY fish at 180 dah. IVF in vitro fertilization. Values are presented as mean ± SD. Different letters above the error bar indicate statistically significant differences at *P* < 0.05 as determined by one-way ANOVA followed by the Tukey test for multiple comparisons. dah days after hatching.
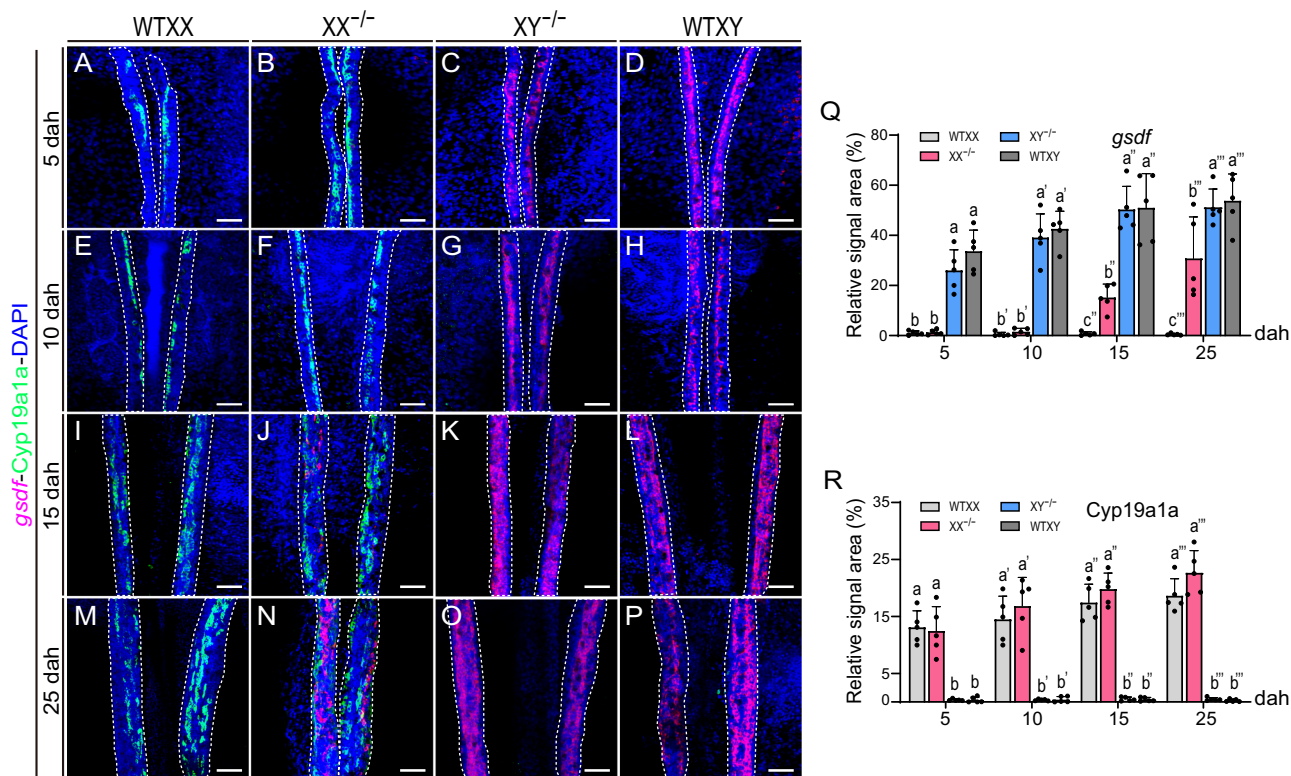
Fig. 5 | *gsdf* and Cyp19a1a expression in early gonads of *cyp11a1*⁻/⁻ XX and *cyp11a1*⁻/⁻ XY fish. A–P Combined whole-mount fluorescence in situ hybridization for *gsdf* (red fluorescence) and immunofluorescence for Cyp19a1a (green fluorescence) in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and *cyp11a1*⁻/⁻ XY fish at 5, 10, 15, 25 dah. The nuclei were stained with DAPI (blue fluorescence). Scale bar, 50 μm. White dash lines outline the gonads. **Q**, **R** Quantification of relative *gsdf* (Q) and Cyp19a1a (R) positive signal area in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and *cyp11a1*⁻/⁻ XY fish at 5, 10, 15, and 25 dah (*n* = 5/genotype). Values are presented as mean ± SD. Different letters above the error bar indicate statistically significant differences at *P* < 0.05, as determined by one-way ANOVA followed by the Tukey test for multiple comparisons. dah days after hatching.

XX fish, we checked the expression of the female germ cell marker gene *foxl3*[35] at 15 dah by FISH combined with Vasa antibody staining. The results showed that even though the number of *foxl3*-positive germ cells in the gonads of the *cyp11a1*⁻/⁻ XX was greatly decreased compared with that in the gonads of the WT XX fish, there were still some *foxl3*-positive germ cells exist, indicating the existence of female germ cells in the gonads of the *cyp11a1*⁻/⁻ XX at 15 dah (Fig. 6A–G).

Transcriptome sequencing was performed to investigate the global gene expression profile in the gonads of the *cyp11a1*⁻/⁻ XX fish at 15 dah. Totally, we detected 1385 DEGs (|log₂FC|≥1, *P* < 0.05) between the gonads of the WT XX and WT XY fish, of which 871 and 514 genes were highly expressed in XX and XY gonads, respectively (Supplementary Fig. 4A). Clustering gene expression analysis revealed similar expression pattern of these DEGs in the gonads of the WT XX and *cyp11a1*⁻/⁻ XX fish (Fig. 6H). The delayed *gsdf* upregulation, retained *foxl3*-positive germ cells, and ovary-like global gene expression pattern demonstrated that the gonads of the *cyp11a1*⁻/⁻ XX fish experienced an ovary-like stage.

Transdifferentiation of germ cells from female to male during sex reversal in *cyp11a1*⁻/⁻ XX fish

Two possible fates of female germ cells exist in the early gonads of *cyp11a1*⁻/⁻ XX fish during sex reversal: either undergo apoptosis or transdifferentiate into male germ cells (Fig. 6I). To determine the exact germ cell fate, we first analyzed the gonadal transcriptome of the *cyp11a1*⁻/⁻ XX and WT XX fish at 15 dah. We detected 403 upregulated and 629 downregulated genes in the gonads of the *cyp11a1*⁻/⁻ XX fish and WT XX fish (Fig. 6J). Go enrichment analysis showed that the upregulated genes were mainly involved in chemokine activity, chemokine receptor binding, myofibril, contractile fiber, and cytokine activity (Fig. 6K). These genes are mainly related to immune

and inflammatory responses. Notably, no apoptosis-related gene was significantly upregulated and enriched (Supplementary Fig. 4B and Supplementary Table 1). The downregulated genes are mainly involved in phosphatidic acid biosynthesis and metabolism, meiosis, thyroid hormone response, and hepoxilin metabolic process (Fig. 6K and Supplementary Table 2). Detailed gene expression analysis revealed decreased expression of genes related to germ cell proliferation (*pcna*, *ccnf*, *ccnb3*, *plk1*, *mis18bp1*, *kif20a*, and *mastl*) and meiosis (*rec8a*, *dmc1*, and *meioc*) (Fig. 6L). TUNEL staining was also performed to evaluate the apoptotic feature in the gonads of *cyp11a1*⁻/⁻ XX fish. Consistent with the transcriptome data, we did not detect any increase of apoptosis in the gonads of *cyp11a1*⁻/⁻ XX fish at 15 dah (Fig. 6M–U). In tilapia, *foxl3* has been proven to determine germ cell fate by antagonizing with *dmrt1*[35]. Therefore, we checked the colocalization of *foxl3* and Dmrt1 in the gonads of the *cyp11a1*⁻/⁻ XX fish by FISH combined with IF. The results showed that both *foxl3* and Dmrt1 were expressed in the gonads of the *cyp11a1*⁻/⁻ XX fish at 15 dah, and they were colocalized in some germ cells (Fig. 6V–A'). These results demonstrate that the female germ cells in the gonads of the *cyp11a1*⁻/⁻ XX fish did not undergo apoptosis, but directly transdifferentiated into male germ cells during sex reversal.

P5 and E2 treatment rescued the sex reversal of *cyp11a1*⁻/⁻ XX fish

The direct product of Cyp11a1 (P5) and two main female hormones (E2, DHP) were used to rescue the sex reversal of *cyp11a1*⁻/⁻ XX fish. FISH results from fish at 25 dah showed that both P5 and E2 treatment rescued the sex reversal of *cyp11a1*⁻/⁻ XX fish as indicated by the disappearance of *gsdf* expression in the gonads (Fig. 7A–E), while DHP treatment failed to rescue the sex reversal of *cyp11a1*⁻/⁻ XX fish (Fig. 7F). Histological analyses showed that a large number of oocytes were present in the gonads of WT XX fish, as well as in P5- and E2-treated *cyp11a1*⁻/⁻ XX fish at 90 dah, while no oocytes

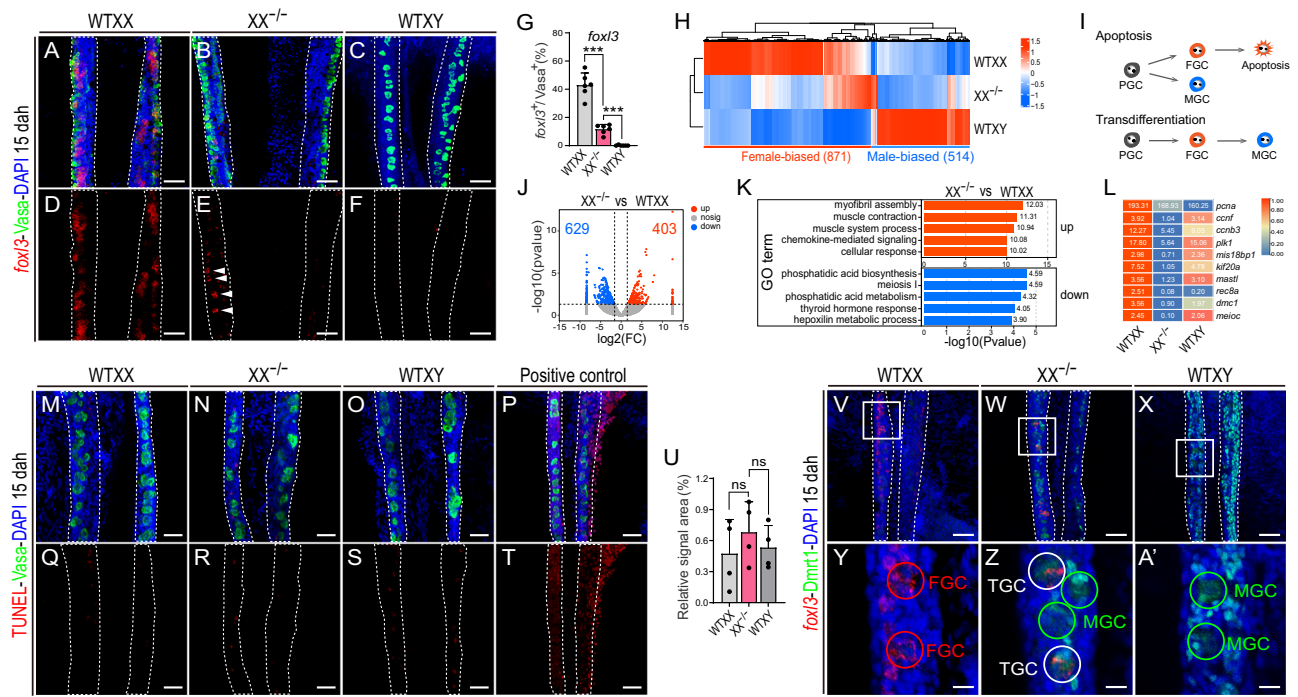**Fig. 6 | Transdifferentiation of germ cells from female to male in the gonads of *cyp11a1*⁻/⁻ XX fish during sex reversal. A–F** Combined whole-mount fluorescence in situ hybridization for *foxl3* (red fluorescence) and immunofluorescence for Vasa (green fluorescence) in the gonads of the WT XX, WT XY, and *cyp11a1*⁻/⁻ XX fish at 15 dah. The nuclei were stained with DAPI (blue fluorescence). Arrowheads indicate the *foxl3*-positive germ cell. Scale bar, 50 μm. White dash lines outline the boundary of the gonads. **G** Quantification of *foxl3*-positive germ cell in the gonads of the WT XX, WT XY, *and cyp11a1*⁻/⁻ XX fish at 15 dah (*n* = 6/genotype). **H** Hierarchical clustering of gene expression in the gonads of the WT XX, WT XY, and *cyp11a1*⁻/⁻ XX fish at 15 dah. The color from blue to red indicates the expression of genes from low to high. **I** Schematic diagram displaying two possible fates of the female germ cells (FGC) in the early gonads of *cyp11a1*⁻/⁻ XX fish: undergo apoptosis or transdifferentiate into male germ cells (MGC). **J** Volcano plot of DEGs in the gonads of the WT XX and *cyp11a1*⁻/⁻ XX fish. **K** GO enrichment analysis of the DEGs in the gonads of the WT XX and *cyp11a1*⁻/⁻ XX fish. **L** The expression of genes related to mitosis (*pcna*, *ccnf*, *ccnb3*, *plk1*, *mis18bp1*, *kif20a*, and *mastl*) and meiosis (*rec8a*, *dmc1*, and *meioc*) in the gonads of the WT XX, WT XY, and *cyp11a1*⁻/⁻ XX fish. The

number inside the square is the RPKM value of genes in each sample. The color from blue to red represents the expression of genes from low to high in each sample. **M–T** TUNEL staining in the gonads of the WT XX, WT XY, and *cyp11a1*⁻/⁻ XX fish at 15 dah. The positive signal corresponds to red fluorescence. Antibody against Vasa was used to stain the germ cell (green fluorescence), and the nuclei were stained with DAPI (blue fluorescence). White dash lines outline the boundary of the gonads. Scale bar, 50 μm. **U** Quantification of TUNEL-positive signal area in the gonads of the WT XX, WT XY, and *cyp11a1*⁻/⁻ XX fish (*n* = 4/genotype). Values are presented as mean ± SD. Differences were determined by one-way ANOVA followed by Tukey test for multiple comparisons. ns not significant. **V–A'** Colocalization of *foxl3* and Dmrt1 in the gonads of the WT XX, WT XY, and *cyp11a1*⁻/⁻ XX fish at 15 dah. The nuclei were stained with DAPI (blue fluorescence). White dash lines outline the boundary of the gonads. FGC female germ cells express *foxl3* (red fluorescence), MGC male germ cells express Dmrt1 (green fluorescence), TGC germ cells undergoing transdifferentiation expression both *foxl3* and Dmrt1. Scale bar: **V–X** 50 μm; **Y–A'** 12.5 μm. dah days after hatching.
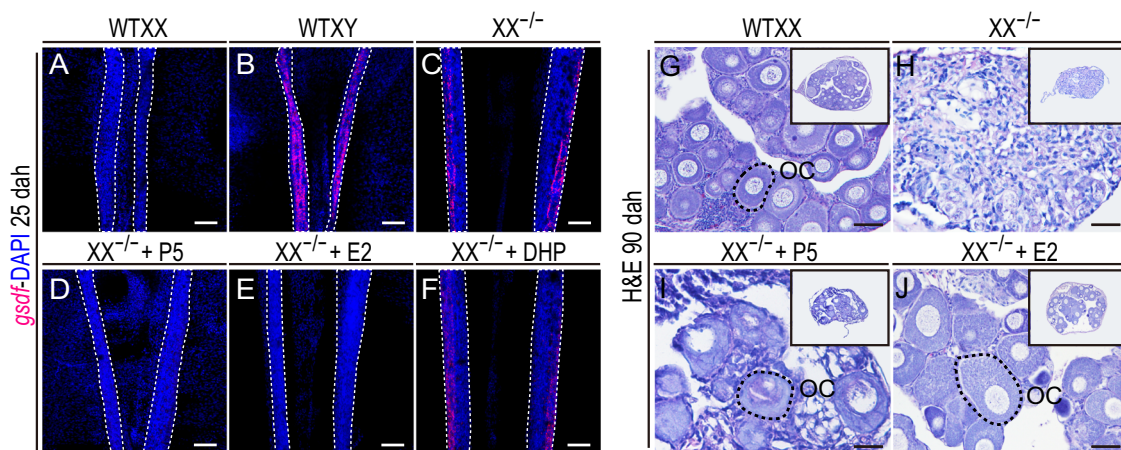

Fig. 7 | P5 or E2 treatment rescued the sex reversal in *cyp11a1*⁻/⁻ XX fish. A–F Whole-mount fluorescence in situ hybridization of *gsdf* in the gonads of the WT XX, WT XY, *cyp11a1*⁻/⁻ XX, and P5, E2, or DHP-treated *cyp11a1*⁻/⁻ XX fish at 25 dah. The positive signal corresponds to red fluorescence. The nuclei were stained

with DAPI (blue fluorescence). White dash lines outline the boundary of the gonads. Scale bar, 50 μm. **G–J** H&E staining of gonads from WT XX, *cyp11a1*⁻/⁻ XX, and P5 or E2-treated *cyp11a1*⁻/⁻ XX fish at 90 dah. OC oocytes. Scale bar: **G, I, J** 40 μm; **H** 20 μm. dah days after hatching.

was found in the gonads of $cyp11a1^{-/-}$ XX fish without treatment, further confirming the rescue effects of P5 and E2 (Fig. 7G–J).

Discussion

cyp11a1 is the only gene currently known to catalyze the production of P5, the precursor of all steroid hormones. In tilapia, only one *cyp11a1* gene exists, as reported in a previous study[11]. In this study, we established a steroid hormone-free genetic model by mutation of *cyp11a1* in tilapia. Even though basal levels of steroid hormones were detected in *cyp11a1* mutant fish, it was more likely due to the non-specificity of the EIA kit. The mutant fish displayed decreased survivability under hypoxic conditions, but they survived under normal water conditions with enough oxygen. Histological analysis, transcriptome analysis, IF, and qPCR analyses revealed female-to-male sex reversal of XX mutants. Detailed FISH and transcriptome analyses captured a transient ovary-like stage at the molecular level and transdifferentiation of germ cells from female to male in the early gonads of the XX mutants.

Fish sex is largely influenced by steroid hormones, especially sex hormones[36–43]. Regarding the way that sex hormones determine sex in fish, there are two theories, which we call the balance theory[36,37] and the absence theory[40,41]. The former holds that fish sex is determined by the ratio of estrogen to androgen in the gonads during sexual differentiation, while the latter believes that fish sex is determined by the presence or absence of estrogen during sex differentiation. The discrepancy between the two lies in whether androgen is involved in sex determination. With the deepening of research in fish sex determination, the absence theory has been widely supported. In hermaphroditic fish, sex change from male-to female (protandrous) or from female to male (protogynous) is associated with the upregulation or downregulation of estrogen levels, respectively[44–49]. In gonochoristic fish, it has been suggested that *cyp19a1a* is expressed in the gonads of genetic female fish at the critical window of sex determination[28,29,39]. Blocking estrogen synthesis through AI treatment[12–17,50–52] or gene mutation[16,18–20,22,30,53] leads to female-to-male sex reversal, and E2 treatment[41,42,54] leads to male-to-female sex reversal in many fish species. In this study, we established a steroid hormone-free genetic model by mutation of *cyp11a1* in tilapia. We found that the estrogen- and androgen-deprived *cyp11a1* mutant XX and XY fish developed as males, consistent with the phenotype of all-male development in zebrafish after *cyp11a2* mutation[9,10]. Rescue experiments showed that E2 treatment rescued the sex reversal of the *cyp11a1* mutant XX fish, indicating that it is the presence or absence of estrogen determines the sex. The facts that the testicular fate of the gonads of *cyp11a1* mutant XY fish was not affected and the gonads of *cyp11a1* mutant XX fish could still transform into testes even in the absence of androgens supports the point that androgen is not involved in fish sex determination. Most importantly, no steroidogenic/androgenic enzyme genes were found to be expressed in the WT XY gonads of tilapia by IHC, IF, qPCR, and transcriptomic analyses[28,29], that explains why the testicular fate of *cyp11a1* mutant XY fish was not affected in the present study. These results support the absence theory. Even though androgen treatment has been reported to result in masculinization or female-to-male sex reversal in some fish species, such as Japanese flounder, tilapia, rainbow trout, medaka, zebrafish, and orange-spotted grouper[17,50,55–58], no sex reversal has been reported when androgen synthesis or androgen receptor is disrupted[59–66]. The androgen-induced sex reversal in fish is thought to be achieved by inhibition of female pathway genes[67] or induction of male pathway genes[56]. In this study, we observed a continuous expression of *cyp19a1a* in the gonads of the *cyp11a1* mutant XX fish. The expression of *cyp19a1a* was eliminated by 11-KT treatment. Luciferase assay revealed direct inhibition of androgen to the *sf1*-activated *cyp19a1a* transcription via AR1 and AR2 and synergistic inhibitory effects of androgen and *dmrt1* to *cyp19a1a* transcription. Taken together, our results confirm that it is estrogen, not androgen, that determines the sex of fish. The androgen-induced sex reversal in fish can be mediated by the inhibition of Sf1-activated *cyp19a1a* transcription.

Even though the role of estrogen in ovary development has been widely accepted, the dependence of early ovary differentiation on estrogen seems to vary among different fish species. In medaka, *cyp19a1a* expression is detected after the initiation of oogenesis[25,26]. Blocking estrogen synthesis through AI treatment leads to female-to-male sex reversal but does not affect early oogenesis[26]. The gonad of *cyp19a1a* mutant female medaka first developed into an ovary but gradually transformed into a testis with the degeneration of ovarian tissue[21]. In zebrafish, blocking estrogen synthesis by mutation of *cyp19a1a*, *cyp17a1*, or *cyp11a2* leads to all-male development but the juvenile ovary and oocyte-like germ cells formed at early developmental stage[10,18,22]. These results demonstrate that sex reversal caused by estrogen deficiency in medaka and zebrafish experience an ovary stage. Gonad differentiation is a continuous process involving expression changes of a large number of genes. In tilapia, the molecular differentiation of the gonads initiates around 5 dah, and the first sign of morphological differentiation of the gonads occurs around 23 to 26 dah[28]. Previously, the morphological features of gonads or the expression of few marker genes were used to characterize the sex reversal process of *cyp19a1a*, *cyp17a1* mutant fish in tilapia[20,30], which ignored the global gene expression profiles. In this study, we characterized the sex reversal process of the *cyp11a1* mutant XX fish comprehensively at a molecular level. Transcriptome analysis showed that at 15 dah, the global gene expression pattern in the gonads of the mutant XX fish was relatively more similar to those of the WT XX fish, but at 90 dah, it was relatively more similar to those of the WT XY fish. Consistently, FISH analyses revealed that the expression of *gsdf* was detected in the gonads of WT XY fish at 5 dah, but it was not detected in the gonads of the mutant XX fish until 10–15 dah. Even at 15 dah, *foxl3* was still expressed in the gonads of the mutant XX fish. These results demonstrate that the steroid hormone-deprived sex reversal of *cyp11a1* mutant XX fish in tilapia experiences an ovary-like stage at a molecular level. The fact that E2 treatment rescued the sex reversal of the *cyp11a1* mutant XX fish in this study indicates that the cause of the sex reversal of the *cyp11a1* mutant XX fish is estrogen deficiency. Whether the sex reversal caused by estrogen deficiency experience an ovary-like stage at molecular level in other genetic model in tilapia and other fish species is worthy of investigation.

Transdifferentiation describes a direct transition of one differentiated cell type to another differentiated cell type during development[68], and it has been observed in adult gonads in both mammals and fishes during sex reversal. In mouse, Foxl2 ablation in adults leads to transdifferentiation of ovarian granulosa and theca cells into testicular Sertoli-like and Leydig-like cells[69], and Dmrt1 ablation leads to transdifferentiation of Sertoli cells into granulosa cells[70]. In tilapia, AI treatment of XX fish from 90 to 180 dah induces the successful transition of the differentiated ovary into the testis, during which transdifferentiation is observed in somatic cells[24]. Whether germ cells undergo transdifferentiation in the early gonads during sex reversal is worth investigating. In this study, we captured a transient ovary-like stage and witnessed the existence of *foxl3*-positive female germ cells in the early gonads of the steroid hormone-deprived *cyp11a1* mutant XX fish. In theory, these *foxl3*-positive female germ cells have two different fates during sex reversal: either undergo apoptosis or transdifferentiate into male germ cells. Detailed gene expression analysis of apoptosis-related genes and TUNEL staining excluded the possibility of apoptosis in these cells. Recently, we have proved that in tilapia, *foxl3* is expressed in female germ cells but not in male germ cells. *dmrt1* is expressed both in male germ cells and somatic cells but not in female germ cells and somatic cells. The sexual fate of tilapia germ cells is determined by the antagonistic interaction of *dmrt1* and *foxl3*[35]. In this study, we detected colocalization of *foxl3* and *dmrt1* in germ cells in the early gonads of the mutant XX fish, which has never been detected in the WT XX and WT XY gonads. The germ cells expressing both *foxl3* and *dmrt1* may be undergoing transdifferentiation. Even though we cannot rule out the possibility that male germ cells in the early gonads of *cyp11a1* mutant XX fish can be directly differentiated from primordial germ cells, we demonstrate that germ cells can undergo transdifferentiation at the early stage of steroid hormone-deprived sex reversal in this study.
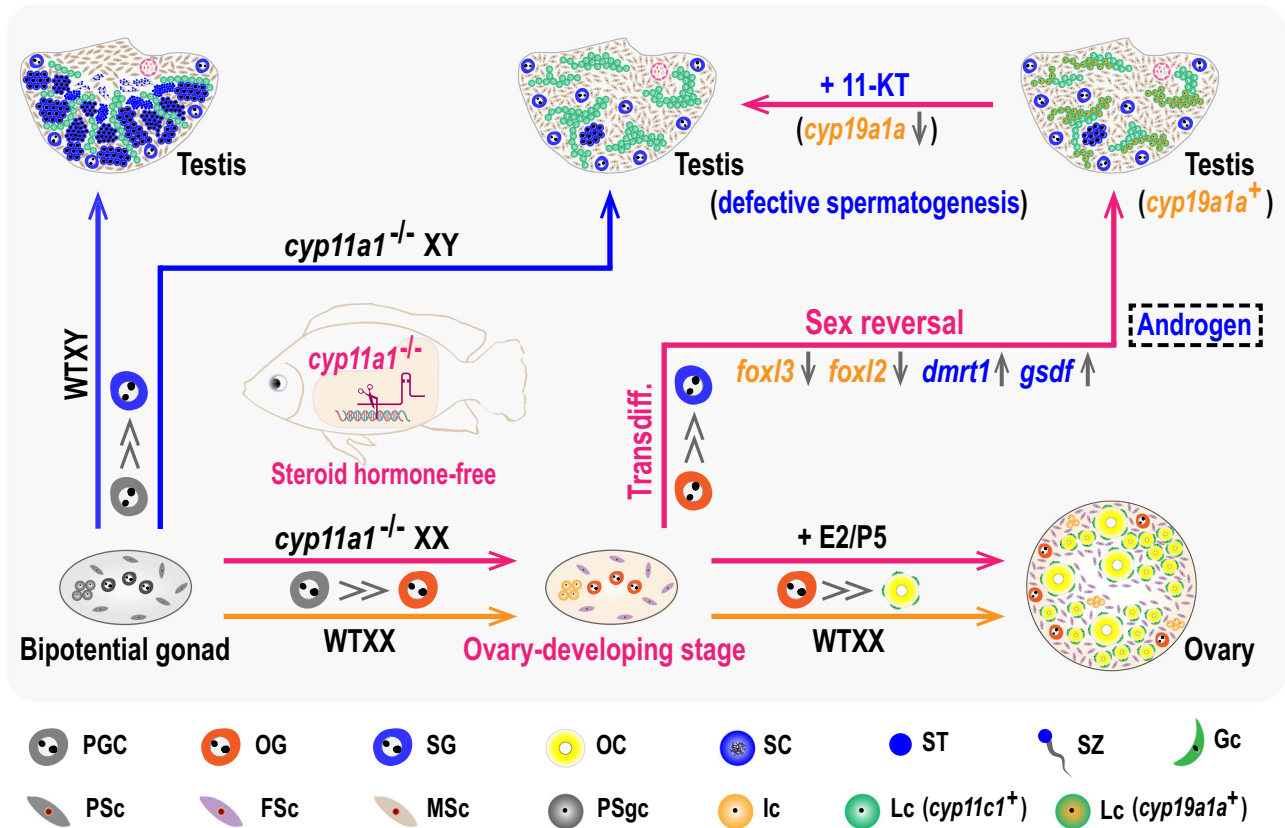
Fig. 8 | Schematic diagram displaying the gonadal development of steroid hormone-free tilapia. A steroid hormone-free tilapia model was established by mutation of *cyp11a1*. Both XX and XY *cyp11a1* mutants developed as infertile males due to defects in spermatogenesis. The gonads of the *cyp11a1* mutant XX fish first developed toward the ovary but shifted to the testis during 10 to 15 dah. Both P5 and E2 treatment rescued the ovary development of the *cyp11a1* mutant XX fish. Due to the simultaneous loss of androgen, *cyp19a1a* was continuously expressed in the gonads of the *cyp11a1* mutant XX fish. PGC primordial germ cells, OG oogonia, SG spermatogonia, OC oocytes, SC spermatocytes, ST spermatids, SZ spermatozoa, Gc granulosa cells, PSc pre-supporting cells, FSc female supporting cells, MSc male supporting cells, PSgc pre-steroidogenic cells, Ic interstitial cells, Lc Leydig cells.

In summary, we established a steroid hormone-free genetic model in tilapia by mutation of *cyp11a1*. We showed that the gonads of steroid hormone-free tilapia developed into testis in both XX and XY fish with defective spermatogenesis. The gonads of the mutant XX fish first developed toward the ovary, but shifted to the testis during 10–15 dah. Due to the simultaneous loss of androgen and estrogen, *cyp19a1a* was continuously expressed in the testis of the mutant XX fish. Both P5 and E2 treatment rescued the sex reversal of the mutant XX fish (Fig. 8). This study revealed a transient ovary-like stage and transdifferentiation of germ cells from female to male in the early gonads of tilapia after steroid hormone deprivation.

Methods
Animals
Nile tilapia (*O. niloticus*) used in this study was first introduced by Prof. Nagahama (Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki, Japan) and kept in aerated recirculating freshwater tanks at 26 °C under a natural photoperiod in our lab. Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

Establishment of *cyp11a1* mutant line
The sequence of tilapia *cyp11a1* (Gene ID: 100692956) was obtained from NCBI. The target site with a *Sau*96I restriction site (underlined) was designed in the 4th exon of *cyp11a1* using the online software ZiFit (http://zifit.partners.org/ZiFiT/). The guide RNA and Cas9 mRNA were prepared as previously reported[32]. Embryos at the one-cell stage were co-injected with gRNA and Cas9 mRNA with a final concentration of 500 and 1000 ng/µl, respectively. After mutation screening by polyacrylamide gel electrophoresis (PAGE) and Sanger sequencing, F0 chimeric adult XY males were crossed with wild-type (WT) XX females to produce F1 progeny. Siblings in F1 carrying a 22 bp deletion at the same locus were incrossed to produce F2 progeny. *Sau*96I digestion and PAGE was used for mutation screening in the F2 population. The genetic sex of each fish was identified by a sex-linked marker as described previously[31]. Primers used for gRNA synthesis, mutation screening, and genetic sex identification are listed in Supplementary Table 3. After mutation screening, the proportion of *cyp11a1* homozygous mutant (*cyp11a1*$^{-/-}$) fish in the F2 population at 5, 15, 25, 35, and 90 dah was calculated ($n = 4$ populations at each time point, more than 48 fish were randomly selected in each population).

Measurement of suffocation point
After genotyping, the 90-dah-old WT XY and *cyp11a1*$^{-/-}$ XY fish ($n = 6$/genotype) with similar body size (body length: WT XY 6.63 ± 0.34 cm, *cyp11a1*$^{-/-}$ XY 6.60 ± 0.30 cm; body weight: WT XY 10.37 ± 0.90 g; *cyp11a1*$^{-/-}$ XY 10.35 ± 0.78 g) were kept in a tank containing 20 L of water at 26 °C. Oxygen in the water was deprived by continuous filling of nitrogen. The suffocation point, which refers to the minimal oxygen concentration at which the fish loses balance, was determined by using the average value of six fish after measurement of the dissolved oxygen concentration when each fish loses balance. The dissolved oxygen concentration was measured by HQ30D portable dissolved oxygen meter (HACH, Colorado, USA).

Measurement of steroid hormones
Enzyme immunoassay (EIA) kit was used to measure the serum P5 (Spbio, Wuhan, China) and cortisol level of WT XY and *cyp11a1*$^{-/-}$ XY fish, and

serum estradiol (E2), 17,20 β-dihydroxy-4-pregnen-3-one (DHP), testosterone (T) and 11-ketotestosterone (11-KT) (Cayman, Michigan, USA) level of WT XX, WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 90 dah. After anesthesia with MS-222 (250 mg/L, Sigma-Aldrich, Missouri, USA), blood was collected from the caudal vasculature of fish from each genotype (serum from three fish as one sample, $n \geq 3$/genotype) and placed at 4 °C overnight to allow clot. The serum was separated by blood centrifugation (1000×g, 10 min, 4 °C) and stored at −80 °C. EIA was performed according to the manufacturer's instructions. All samples were run in triplicate on one 96-well plate to ensure the comparability of each assay.

Quantitative real-time PCR
Twelve tissues (brain, pituitary, gill, heart, spleen, liver, intestine, ovary, testis, kidney, head kidney, and muscle) of WT XX and WT XY fish at 180 dah ($n = 4$/sex) were collected for expression analysis of $cyp11a1$ in different tissues. Gonads of WT XX, WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 90 dah were collected for gene expression analysis of $foxl2$, $dmrt1$, $cyp19a1a$, and $cyp11c1$ ($n = 6$/genotype). Total RNA was isolated using RNAiso Plus (Takara, Tokyo, Japan). RNA quality and concentration were determined using NanoDrop 2000, and ≥500 ng total RNA was used for cDNA synthesis with PrimeScript RT Master Mix Perfect Real Time kit (Takara, Tokyo, Japan). Quantitative real-time PCR (qPCR) was performed using a TB Green Premix Ex Taq II kit (Takara, Tokyo, Japan). All experiments were performed according to the manufacturer's instructions. The value was detected on the StepOne Plus Real-time PCR system (Thermo Fisher Scientific, Massachusetts, USA). Gene expression was normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method[71]. Primers used for qPCR are listed in Supplementary Table 3.

In situ hybridization
In situ hybridization was performed to investigate the cellular location of $cyp11a1$ in gonads and head kidneys. Ovaries, testes, and head kidneys of WT fish at 240 dah were dissected and fixed in 4% PFA (4 °C, overnight). After fixation, the gonads were embedded in paraffin and sliced into 5 μm sections (cross-section) for use. The digoxigenin (DIG)-labeled sense and antisense probes of $cyp11a1$ were prepared as follows: a fragment (604 bp) of $cyp11a1$ were amplified from cDNA by PCR and recovered. After subjected to TA clone, the plasmid containing T7 promoter and insertion or inverted insertion of $cyp11a1$ fragment were extracted and amplified by PCR. The sense and antisense probes were synthesized by in vitro transcription using the recovered PCR products as templates and a kit containing T7 RNA polymerase and DIG-labeled rNTP mix according to the manufacturer's instructions (Roche, Basel, Switzerland). The in situ hybridization were performed as follows: Briefly, after deparaffinization in xylene, the slides were placed in 100, 90, 80, and 70% ethanol and 1× PBS for rehydration. Then, the slides were fixed in 4% PFA again, and rinsed in 2 mg/mL glycine, 0.1 M TEA, 0.25% acetic anhydride/0.1 M TEA, successively. Next, the slides were placed in pre-hybridization buffer (66% formamide; 10% 20× SSC) at 65 °C for 2 h and in hybridization buffer (60% formamide; 7.5% dextran sulfate; 0.3 M NaCl, 0.02 M Tris-HCL PH 8.0; 0.0025 M EDTA; 1X Denhardt solution) containing tRNA (20 μg/mL) and antisense probe (500 ng/mL) for hybridization (65 °C, 16 h). After hybridization, the slides were washed stringently with 50% formamide/ 2× SSC, 1× SSC, 0.2× SSC, DIG buffer (0.1 M Maleic acid; 0.15 M NaCl; PH 7.4) and blocked in DIG buffer containing 5% BSA at 37 °C for 1 h. After these steps, the slides were incubated with anti-DIG-AP (Roche, Basel, Switzerland) at 4 °C overnight. Finally, the slides were rinsed in a DIG buffer five times and hatched in a detection buffer (1 M Tris-HCL PH 9.5; 5 M NaCl; 1 M MgCL₂) containing NBT/BCIP (Roche, Basel, Switzerland) for signal detection. Images were captured under a BX53 microscope (Olympus, Tokyo, Japan). Primers used for probe preparation are listed in Supplementary Table 3.

Hematoxylin and eosin staining
Gonads from WT XX, WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 90 dah and WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 180 dah were

dissected and fixed in Bouin's solution (24 h, room temperature). After fixation, gonads were embedded in paraffin and sliced into 5 μm sections (middle cross-section) for use. For hematoxylin and eosin (H&E) staining, tissue sections were firstly deparaffinized in xylene and rehydrated in decreasing concentration of ethanol. Then, tissue sections were stained with hematoxylin and eosin successively. Finally, sections were dehydrated in increasing concentration of ethanol and mounted. Images were captured under a BX51 optical microscope (Olympus, Tokyo, Japan). The cross-sectional area of testes of WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 90 and 180 dah was quantified by Image J Pro 1.51 software using default parameters ($n = 6$ fish/genotype). The gonadosomatic index (GSI) of WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 180 dah was calculated as (gonad weight/body weight) × 100% ($n = 6$ fish/genotype).

Papanicolaou staining, mobility test, and fertilization assay
Since no sperm could be squeezed out from the genital pore of $cyp11a1$ mutants at 180 dah, the testes of WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish were dissected and minced with scissors for further Papanicolaou (Pap) staining, mobility test and in vitro fertilization (IVF) assay to check whether fertile sperm was produced in the mutants ($n = 6$/genotype). For Pap staining, the homogenates were firstly fixed in 4% PFA for 10 min then stained with Papanicolaou solution EA50 (Solarbio, Beijing, China) for 3 min. Images were captured under a BX51 optical microscope (Olympus, Tokyo, Japan). For the mobility test, the homogenates were mixed with 20 times the volume of water and analyzed under a Sperm Quality Analyzer (Zoneking Software, Beijing, China). For the IVF assay, the homogenates of WT and mutant fish was used to inseminate eggs from WT XX fish. The embryos were checked at 16 h post-fertilization under Leica M205 FA Stereomicroscope (Leica, Wetzlar, Germany) to assess whether fertilization is successful. The fertilization rate was calculated as (the number of eggs with normal blastoderm at 16 hpf/the total number of eggs) × 100%.

Immunofluorescence
Immunofluorescence (IF) was performed to evaluate the expression of Cyp19a1a, Cyp11c1, and Vasa in the gonads of the WT XX, WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 90 dah ($n = 4$/genotype). Gonads were dissected and fixed in Bouin's solution (24 h, room temperature). After fixation, gonads were embedded in paraffin and sliced into 5 μm sections (cross-section) for use. After deparaffinization and hydration, sections were subjected to antigen retrieval and blocked in donkey serum (37 °C, 1 h). Sections were then incubated with primary antibody (37 °C, 1 h). The rabbit polyclonal antibodies against Cyp19a1a (2 mg/mL, 1:2000) and Cyp11c1 (2 mg/mL, 1:500) were produced by our lab, and their specificity has been verified previously[24,65]. Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:500, Thermo Fisher Scientific, Massachusetts, USA) was used to detect the primary antibodies (37 °C, 40 min). The nuclei were stained with DAPI (1:1000, Sigma-Aldrich, Missouri, USA). Images were captured under the FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Whole-mount fluorescence in situ hybridization and immunofluorescence
Whole-mount fluorescence in situ hybridization (FISH) and IF were combined to track the expression of $gsdf$ and Cyp19a1a in the gonads of the WT XX, WT XY, $cyp11a1^{-/-}$ XX, and $cyp11a1^{-/-}$ XY fish at 5, 10, 15, 25 dah ($n = 5$/genotype) and the expression of $foxl3$ and Dmrt1 in the gonads of the WT XX, WT XY, and $cyp11a1^{-/-}$ XX fish at 15 dah ($n = 6$/genotype). The DIG-labeled antisense probes of $foxl3$ and $gsdf$ were prepared as described in the in situ hybridization section of this study. Primers used are listed in Supplementary Table 3. The rabbit antibodies against tilapia Cyp19a1a (2 mg/mL, 1:2000), Dmrt1 (2 mg/mL, 1:500), and Vasa (2 mg/mL, 1:1000) were produced by our lab, and their specificity has been verified previously[24,35]. After removing the viscera, fish with gonads were fixed in 4% PFA at 4 °C overnight, and placed in 100% methanol at −20 °C for at least 2 h. Then, the fish were placed in 75, 50, 25% methanol, and 1× PBS

successively for rehydration and in a 3% H_2O_2/0.5% KOH medium under light to remove pigment (room temperature, 10 min). After permeabilization in precooled acetone ($-20\,°C$, 30 min), the fish were rinsed four times in 1× PBS and were placed in pre-hybridization buffer (50% formamide; 25% 20× SSC; 0.01 M citric acid; 0.1% Tween-20) at 65 °C for 2 h. Subsequently, the fish were placed in a pre-hybridization buffer containing tRNA (500 μg/mL), heparin (50 μg/mL), and an antisense probe (50–500 ng/mL) for hybridization (65 °C, 16 h). After hybridization, the fish were washed stringently with pre-hybridization buffer, 1× SSC, 0.2× SSC, TN buffer (0.1 M tris-HCL; 0.15 M NaCl; PH 7.5) successively and blocked in TN buffer containing blocking regent (Lot 46925300, Roche, Basel, Switzerland) and 5% donkey serum (37 °C, 1 h). Next, the fish were incubated with anti-DIG-POD (Roche, Basel, Switzerland) at 4 °C overnight. For signal detection, tyramide signal amplification (TSA^{TMR}) was performed according to the manufacturer's instructions (Akoya Biosciences, Marlborough, USA). After TSA amplification, the fish were rinsed four times in 1× PBS and incubated with primary antibody (37 °C, 1 h). Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:500, Thermo Fisher Scientific, Massachusetts, USA) was used to detect the primary antibody (37 °C, 40 min). The nuclei were stained with DAPI (1:1000, Sigma-Aldrich, Missouri, USA). Images were captured under the FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan). The positive signal of *gsdf* and Cyp19a1a was quantified by Image J Pro 1.51 software using default parameters. The zones selected for quantification were in the middle of the gonads. The number of *foxl3*-positive germ cells was quantified manually.

TUNEL staining

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) kit (Roche, Basel, Switzerland) was used to assess the apoptosis in the gonads of the WT XX, WT XY, and *cyp11a1*$^{-/-}$ XX fish at 15 dah ($n = 4$/genotype). Rabbit antibody against tilapia Vasa (2 mg/mL, 1:500) was used to mark the germ cells. The TUNEL staining was conducted as follows: after removing the viscera, fish with gonads were fixed in 4% PFA at 4 °C overnight, and placed in 100% methanol at $-20\,°C$ for at least 2 h. Then, the fish were placed in 75, 50, 25% methanol, and 1× PBS successively for rehydration. After permeabilization in 0.1% triton X-100 (37 °C, 10 min), fish were rinsed four times in 1× PBS and were incubated with TUNEL reaction mixture containing 5 μL enzyme solution and 45 μL label solution (37 °C, 1 h). The positive control was treated with 3 U/mL DNase I (37 °C, 10 min) before reaction mixture incubation. The negative control was incubated only with 50 μL label solution. After TUNEL incubation, the fish were rinsed four times in 1× PBS and blocked in 5% donkey serum (37 °C, 1 h). Fish were next incubated with Vasa antibody (37 °C, 1 h). Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:500, Thermo Fisher Scientific, Massachusetts, USA) was used to detect the primary antibody (37 °C, 40 min). The nuclei were stained with DAPI (1:1000, Sigma-Aldrich, Missouri, USA). Images were captured under the FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan). The positive signal of TUNEL in the gonads was quantified using Image J Pro 1.51 software.

Transcriptome analyses

The expression of *cyp11a1* in the gonads of tilapia at 5, 7, 20, 30, 40, 90, and 180 dah was determined by analyzing the gonadal transcriptome in our lab[29,72]. To evaluate the global gene expression in the gonads of the *cyp11a1* mutants, the gonads of the WT XX, WT XY, and *cyp11a1*$^{-/-}$ XX fish at 15 dah (pooled sample, 30 fish/genotype) and the gonads of the WT XX, WT XY, *cyp11a1*$^{-/-}$ XX, and *cyp11a1*$^{-/-}$ XY fish at 90 dah (pooled sample, 4 fish/genotype) were collected. After total RNA was extracted, mRNA was enriched by Oligo(dT) beads. Then the enriched mRNA was reversely transcribed into cDNA and disrupted into short fragments for cDNA library construction. The resulting cDNA library was sequenced on Illumina Novaseq 6000 by Gene Denovo Biotechnology Co. (Guangzhou, China). Raw reads were filtered by fastp to remove adapters and low-quality reads. Reads were submitted to the NCBI SRA database (Accession number: PRJNA1074672). Clean reads from each library were aligned to the reference genome (https://www.ncbi.nlm.nih.gov/datasets /genome/ GCF_001858045.2/) using HISAT2 with default parameters[73]. The reads per kb per million mapped reads (RPKM) method was used to quantify the gene expression level. Pearson correlation analysis of gene expression in the gonads of WT XX, WT XY, *cyp11a1*$^{-/-}$ XX, and *cyp11a1*$^{-/-}$ XY fish at 90 dah was performed to evaluate the sample relationship. It was calculated by sample relationship analysis tools on the Omicshare web server (https://www.omicshare.com) based on the global gene expression of each sample. Differential expression analysis was performed by edgeR software[74]. The p values was calculated according to the suggested BCV (square-root-dispersion) value (BCF = 0.1). The $\log_2$ (FC, fold change) ≥ 2, $P < 0.01$ (90 dah), or $P < 0.05$ (15 dah) were set as the threshold for significantly differential expression. GO enrichment analysis and clustering gene expression analysis were also finished on the Omicshare web server (https://www.omicshare.com).

Rescue experiment and androgen treatment

The direct product of Cyp11a1 (P5) and two main female hormones (E2, DHP) were used to rescue the sex reversal of *cyp11a1*$^{-/-}$ XX fish. After randomized grouping, the fish with mixed genotypes were firstly raised in aerated water containing 100 μg/L P5, E2, or DHP or the same volume of ethanol (ctrl group) from 5 to 25 dah. The rescue effect of P5, E2, and DHP was first evaluated by FISH at 25 dah using *gsdf* antisense probe ($n = 5$/genotype). After evaluation, the successfully rescued groups were subjected to treatment with the same hormone until 90 dah. The treatment of fish from 25 to 90 dah was conducted with a diet containing P5 or E2 at a dosage of 50 μg/g. The gonad phenotype of rescued *cyp11a1*$^{-/-}$ XX fish was evaluated by histological analysis ($n = 5$/genotype). Androgen treatment of *cyp11a1*$^{-/-}$ XX fish was conducted from 75 to 90 dah with a diet containing 11-KT at a dosage of 50 μg/g. The expression of Cyp19a1a was analyzed by IF after treatment ($n = 4$/genotype). The positive signal of Cyp19a1a was quantified by Image J Pro 1.51 software using default parameters. The zones selected for quantification were the whole gonads.

Dual-luciferase reporter assay

The promoter of *cyp19a1a* and the open reading frame of *sf1*, *dmrt1* were cloned into pGL3 and pcDNA3.1 vector, respectively, in our previous study[75,76]. The open reading frame of *ar1* and *ar2* was inserted into *Hind* III and *Xho* I restriction sites of the pcDNA3.1 vector in this study. The Luciferase assay was conducted as follows: HEK293 cells were plated on a 24-well plate at a density of 1×10^6 cells per well. After growing to 70–80% confluence, cells were transfected with pGL3-*cyp19a1a* (250 ng/well), pcDNA3.1-*sf1*, -*dmrt1*, -*ar1*, -*ar2* (100 ng/well), and pRL-TK (internal control, 50 ng/well) constructs using Lipofectamine 2000 (Invitrogen, California, USA). At 12 h post-transfection, 200 ng 11-KT was added to each well to activate the ARs. At 48 h post-transfection, the cells were harvested and lysed. The firefly luciferase enzyme activity (pGL3-) was measured and normalized to the *Renilla* luciferase enzyme activity (pRL-TK) according to the manufacturer's instruction (Promega, Wisconsin, USA). Primers used for plasmid construction are listed in Supplementary Table 3.

Statistics and reproducibility

Values are presented as mean ± SD. A two-tailed independent Student's *t*-test was used to determine the differences between the two groups. One-way ANOVA, followed by Tukey multiple comparison, was used to determine the significance of differences in more than two groups. All analyses were performed using SPSS 22.0 (IBM, New York, USA). $P < 0.05$ was used as a threshold for statistically significant differences. Experiments except transcriptome sequencing in this study were performed twice to ensure the reproducibility.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

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Author contributions
D.W. and H.X. conceived and designed the experiments; H.X., L.W., S.Y., H.M., Z.X., F.W., and J.W. performed the experiments; H.X. and W.T. analyzed the data, interpreted the results, and drafted the manuscript;

D.W. critically edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information