Blockage of androgen and administration of estrogen induce transdifferentiation of testis into ovary

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Abstract

Induction of sex reversal of XY fish has been restricted to the sex undifferentiated period. In the present study, differentiated XY tilapia were treated with trilostane (TR), metopirone (MN) and glycyrrhetic acid (GA) (inhibitor of 3β -HSD, Cyp11b2 and 11 β -HSD, respectively) alone or in combination with 17 β -estradiol (E2) from 30 to 90 dah (days after hatching). At 180 dah, E2 alone resulted in 8.3%, and TR, MN and GA alone resulted in no secondary sex reversal (SSR), whereas TR + E2, MN + E2 and GA + E2 resulted in 88.3, 60.0 and 46.7% of SSR, respectively. This sex reversal could be rescued by simultaneous administration of 11-ketotestosterone (11-KT). Compared with the control XY fish, decreased serum 11-KT and increased E2 level were detected in SSR fish. Immunohistochemistry analyses revealed that Cyp19a1a, Cyp11b2 and Dmrt1 were expressed in the gonads of GA + E2, MN + E2 and TR + E2 SSR XY fish at 90 dah, but only Cyp19a1a was expressed at 180 dah. When the treatment was applied from 60 to 120 dah, TR + E2 resulted in 3.3% of SSR, MN + E2 and GA + E2 resulted in no SSR. These results demonstrated that once 11-KT was synthesized, it could antagonize E2-induced male-to-female SSR, which could be abolished by simultaneous treatment with the inhibitor of steroidogenic enzymes. The upper the enzyme was located in the steroidogenic pathway, the higher SSR rate was achieved when it was inhibited as some of the precursors, such as androstenedione, testosterone and 5 α -dihydrotestosterone, could act as androgens. These results highlight the key role of androgen in male sex maintenance.

Key Words

- ▶ androgen
- ▶ 17 β -estradiol
- ▶ sex maintenance
- ▶ secondary sex reversal of XY fish

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Introduction

In fish, sex is determined by both genetic and environmental factors. The latter, especially sex steroid hormones, could even override the genetic factors and change the fate of the gonad. In teleost, endogenous

estrogens are synthesized in the ovary during the critical period of molecular sex differentiation and act as the natural inducer of ovarian differentiation (Nagahama 2000, Tao *et al.* 2013). In females, treatment with aromatase

inhibitors (AI), before and during the period of molecular sex differentiation, induces primary sex reversal (PSR) of the undifferentiated ovary into testis (Piferrer *et al.* 1994, Guiguen *et al.* 1999, Kwon *et al.* 2000, Suzuki *et al.* 2004). Estrogens also play key roles in female sex maintenance. Long-term treatment with AI even induces secondary sex reversal (SSR) of the morphologically differentiated ovary into functional testis (Paul-Prasanth *et al.* 2013, Takatsu *et al.* 2013, Sun *et al.* 2014). In species with natural sex reversal (NSR), the serum 17 β -estradiol (E2) level decreases rapidly during female-to-male sex reversal in protogynous fishes (Nakamura *et al.* 1989, Kokokiris *et al.* 2006, Nozu & Nakamura 2015), whereas it increases during male-to-female sex reversal in protandrous fishes (Chang & Yueh 1990, Guiguen *et al.* 1993, Chang *et al.* 1994). In contrast, treatment with AI induces female-to-male sex reversal in protogynous fishes (Bhandari *et al.* 2004a,b, 2005, Benton & Berlinsky 2006) and blocks male-to-female NSR in protandrous fish (Lee *et al.* 2001, 2002).

Many studies have demonstrated that male-to-female PSR could be induced, before and during molecular sex differentiation by the administration of exogenous estrogens in teleosts, including tilapia (Tayaman & Shelton 1978, Guiguen *et al.* 1999, Gennotte *et al.* 2014), medaka (Yamamoto 1953, Kobayashi & Iwamatsu 2005), pufferfish (Lee *et al.* 2009), trout (Guiguen *et al.* 1999, Vizziano *et al.* 2007, Vizziano-Cantonnet *et al.* 2008), fathead minnow (Watanabe *et al.* 2009), pejerrey (Strüssmann *et al.* 1996, Pérez *et al.* 2012), mahseer, common carp and catfish (Singh 2013). Nevertheless, once the gonad is morphologically differentiated, it is very difficult or even impossible to induce sex reversal of genetic male by administration of estrogens. During this period, an important fact is that the steroidogenic enzymes genes, such as *cyp11a1* (cytochrome P450, family 11, subfamily A, polypeptide 1), *3 β -HSD* (3 β -hydroxysteroid dehydrogenase/isomerase), *cyp17a* (cytochrome P450, family 17 and subfamily A), especially *cyp11b2* (cytochrome P450, family 11, subfamily B, polypeptide 2), the key enzyme for 11-ketotestosterone (11-KT, the main androgen in most teleosts) synthesis, started to get expressed in Leydig cells of the testis, and consequently, the androgen level was significantly upregulated in teleost (Borg 1994, Kobayashi *et al.* 1998, Nakamura *et al.* 1998, Vizziano *et al.* 2007, Pérez *et al.* 2012). In tilapia, gonadal morphological differentiation occurs at approximately 23–26 dah (Nakamura & Nagahama 1989). These enzymes *Cyp11a1/cyp11a1*, *Cyp17a1/cyp17a1* and *Cyp11b2/cyp11b2* have not been detected until approximately at 30 dah in the testis

of the XY fish by immunohistochemistry (IHC) and transcriptome analyses (Kobayashi *et al.* 1996, Nagahama 2000, Bhandari *et al.* 2006, Ijiri *et al.* 2008, Tao *et al.* 2013). It is well accepted that endogenous androgens are critical for testicular morphological differentiation (Pérez *et al.* 2012) and spermatogenesis (Miura *et al.* 1991, Cavaco *et al.* 2001, Zhang *et al.* 2010). Therefore, it would be interesting to examine whether endogenous androgens in the morphologically differentiated testis antagonize exogenous estrogens-induced male-to-female SSR in XY fish.

The Nile tilapia (*Oreochromis niloticus*) is a good species for the study of sex differentiation. It is a gonochoristic fish with an XX/XY sex determination system. In this study, we treated XY tilapia with trilostane (TR), metopirone (MN) and glycyrrhetic acid (GA) (inhibitor of 3 β -HSD, Cyp11b2 and 11 β -HSD (11 β -hydroxysteroid dehydrogenase), respectively) alone or in combination with E2 from 30 to 90 dah (Marandici & Monder 1993, Villeneuve *et al.* 2008, Rigel *et al.* 2010). We analyzed the gonad phenotype, rate of sex reversal, gene expression profile and serum hormone levels. Our results indicated that androgens were critical for sex maintenance in males. This experimentally induced SSR of the morphologically differentiated testis into functional ovary was first described in tilapia, even in teleosts.

Materials and methods

Animals

Nile tilapia were reared in recirculating aerated freshwater tanks at 26°C under a natural photoperiod. All-XX progenies were obtained by crossing an XX pseudomale with a normal XX female. All-XY progenies were obtained by crossing an YY supermale with a normal female. 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.

Hormone treatment

Experiment 1: The 30 dah XY fish were divided into TR, MN, GA, E2, TR+E2, MN+E2, GA+E2, TR+E2+11-KT, MN+E2+11-KT, GA+E2+11-KT, TR+E2+MT, MN+E2+MT and GA+E2+MT treatment groups. The XY fish were treated with commercial diet sprayed with

95% ethanol containing the same dose of TR, MN, GA (Sigma-Aldrich, 150 µg/g feed) alone or in combination with E2 (Sigma-Aldrich, 150 µg/g feed) and rescued by administration of 11-KT and MT (17 α -methyltestosterone) (Sigma-Aldrich, 150 µg/g feed) from 30 to 90 dah, and then fed with normal commercial diet till 180 dah, when the fish were sampled. The control XX and XY fish were raised in steroid- and drug-free water and fed with normal commercial diet sprayed with 95% ethanol.

Experiment 2: The 60 dah XY fish were divided into E2, TR+E2, MN+E2 and GA+E2 treatment groups. The XY fish were treated with commercial diet sprayed with 95% ethanol containing 150 µg/g feed of E2 alone or in combination with TR, MN and GA (150 µg/g feed) from 60 to 120 dah, and then fed with normal commercial diet till 180 dah. The control XX and XY fish were raised as described in experiment 1.

The gonads were sampled at 60, 90, 120, 150 and 180 dah and fixed in Bouin's solution for subsequent histological observations. The gonads from the control XX, control XY, TR, MN, GA, E2, TR+E2+11-KT, MN+E2+11-KT, GA+E2+11-KT and sex-reversed TR+E2, MN+E2 and GA+E2 treatment XY fish were sampled for IHC analysis and serum for enzyme immunoassay (EIA) at 90, 120, 150 and 180 dah, and for real-time PCR at 90 and 180 dah. The gonad phenotype and sex reversal rate were determined by histological examination at 180 dah.

Experiment 3: The XX fish were divided into A4 (androstenedione), T (testosterone) and DHT (dihydrotestosterone) experiment groups. The newly hatched XX fry were firstly exposed to A4 (Xiya Reagent, Chengdu, China), testosterone (Sigma-Aldrich) and DHT (Sigma-Aldrich) 50 µg/L water from 0 to 5 dah, and then fed with commercial diet sprayed with 95% ethanol containing A4, testosterone and DHT (50 µg/g diet) until 30 dah. The control XX and XY fish were raised as described previously.

The gonads of the control XX, control XY, A4, testosterone and DHT treatment XX fish were sampled at 30 dah and fixed in Bouin's solution for subsequent histological observations and IHC analysis. The gonad phenotype and sex reversal rate were determined by histological examination and IHC analyses at 30 dah.

IHC analyses

IHC analyses were performed as described previously (Sun *et al.* 2014, Xie *et al.* 2016). Photographs were taken under an Olympus BX51 light microscope (Olympus).

Measurement of serum 11-KT and E2 levels by EIA

Blood samples were collected from the caudal veins of the control XX, XY and treatment XY fish at 90 and 150 dah, and then, kept at 4°C overnight. Serums were collected after centrifugation and stored at -20°C until use. Serum 11-KT and E2 levels were measured using EIA Kit (Cayman) according to the manufacturer's instructions.

Real-time PCR analysis

Gonads were dissected from the control XX, XY and treatment XY fish at 90 dah. Total RNA extraction, reverse transcription and real-time PCR were performed as described previously (Chen *et al.* 2016). The relative abundances of *foxl2* (forkhead box l2), *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a), *sfl* (steroidogenic factor 1), *dmrt1* (doublesex and mab-3-related transcription factor 1), *gsdf* (gonadal soma-derived factor), *cyp11b2*, *cyp26a1* (cytochrome P450, family 26, subfamily A) and *scp3* (synaptonemal complex protein-3) mRNA transcripts were evaluated using the formula: $R = 2^{-\Delta\Delta C_t}$, as described previously (Tao *et al.* 2013, Sun *et al.* 2014). The expression values were normalized as described in our previous study (Chen *et al.* 2016). Primer sequences used for real-time PCR are listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

Results

SSR was successfully induced by treatment of XY fish from 30 to 90 dah

The gonad phenotypes of all experimental fish were examined histologically at 180 dah. All control XY fish developed as male with normal testis and all control XX fish developed as female with normal ovary. Treatment of 30 dah XY fish with TR, MN and GA alone for 60 days resulted in no male-to-female SSR, whereas with E2 alone resulted in 8.3% SSR. Interestingly, TR+E2, MN+E2 and GA+E2 treatment produced 88.3, 60.0 and 46.7% SSR in XY fish, respectively. The SSR was rescued by simultaneous administration of 11-KT or MT ([Table 1](#) and [Supplementary Table 2](#)).

Histologically, in control XY gonad, spermatogonia were observed at 60 dah, primary and secondary spermatocytes appeared at 90 dah, spermatids appeared at 120 dah and spermatozoa appeared 180 dah ([Fig. 1A, B, C and D](#)). The 11-KT rescued XY fish displayed similar testis development

Table 1 Gonadal phenotype and SSR rate at 180 dah.

Treatment	Fish examined	Testis	Ovotestis	Ovary	Sex reversal rate (%)
Control XX	60	0	0	60	0
Control XY	60	60	0	0	0
TR	60	60	0	0	0
MN	60	60	0	0	0
GA	60	60	0	0	0
E2	60	55	5	0	8.3
TR + E2	60	7	14	39	88.3
MN + E2	60	24	18	18	60.0
GA + E2	60	32	10	18	46.7
TR + E2 + 11-KT	60	60	0	0	0
MN + E2 + 11-KT	60	60	0	0	0
GA + E2 + 11-KT	60	60	0	0	0

In the experiment 1, the XY fish were treated with TR, MN and GA alone or in combination with E2 at a concentration of 150 µg/g diet from 30 to 90 dah. 11-KT was used to rescue sex reversal induced by TR+E2, MN+E2 and GA+E2 treatment. The phenotype of fish was determined by gonad histology at 180 dah.

to the control XY fish. Compared with the control XY gonad, delayed spermatogenesis was observed in the TR, MN and GA XY gonad, with only spermatogonia observed at 60 and 90

dah, primary spermatocytes appeared at 120 dah, secondary spermatocytes and spermatids appeared at 180 dah (Fig. 1E, F, G and H). In the TR+E2, MN+E2 and GA+E2 SSR gonad,

Figure 1

Histological observations of gonads from XY control and treatment fish at 60, 90, 120 and 180 dah. In XY control gonad, only spermatogonia were observed at 60 dah, primary and secondary spermatocytes appeared at 90 dah, spermatids appeared at 120 dah and spermatozoa appeared 180 dah (A, B, C and D). In GA XY fish, only spermatogonia were observed at 60 and 90 dah, primary spermatocytes appeared at 120 dah, secondary spermatocytes and spermatids appeared at 180 dah (E, F, G and H). In GA+E2 XY gonad, only testicular tissue was observed at 60 dah; one oocyte appeared adjacent to the blood vessel at 90 dah; several oocytes appeared in the central area of the gonad at 120 dah and only ovarian tissue was observed at 180 dah (I, J, K and L). The SSR induced by E2 treatment alone in XY fish displayed delayed male-to-female sex reversal compared with the GA+E2 XY fish, with testicular tissue observed at 60, 90 and 120 dah, ovotestis with ovarian tissue near blood vessel and testicular tissue opposite to blood vessel observed at 180 dah (M, N, O and P). BV, blood vessel; OC, oocyte; PSC, primary spermatocytes; SG, spermatogonia; SPT, spermatids; SPZ, spermatozoa; SSC, secondary spermatocytes.

Figure 2

Expression of Cyp19a1a, Cyp11b2 and Dmrt1 in the gonad of XX, XY control and XY treatment fish at 60 and 90 dah by immunohistochemistry. The XY fish were treated by TR, MN and GA (150 µg/g diet) alone or in combination with E2 (150 µg/g diet) and rescued by administration of 11-KT (150 µg/g diet) from 30 to 90 dah, and then fed with normal commercial diet. The control fish were raised in steroid-free water and fed with normal commercial diet. Cyp19a1a (A–L and K1–V1) was found to be expressed in the interstitial cells of the gonad of the control XX at 60 dah (A), and control XX (K1), TR+E2 (Q1), MN+E2 (R1) and GA+E2 fish (S1) at 90 dah. Cyp11b2 (M–X and W1–H2) was found to be expressed in the interstitial cells of the gonad of the control XY (N and X1), TR (O and Y1), MN (P and Z1), GA (Q and A2), E2 (R and B2), TR+E2 (S and C2), MN+E2 (T and D2), GA+E2 (U and E2), TR+E2+11-KT (V and F2), MN+E2+11-KT (W and G2) and GA+E2+11-KT XY fish (X and H2) at 60 and 90 dah. Dmrt1 (Y–J1 and I2–T2) was found to be expressed in Sertoli cells of the gonads of the control XY (Z and J1), TR (A1 and K2), MN (B1 and L2), GA (C1 and M2), E2 (D1 and N2), TR+E2 (E1 and O2), MN+E2 (F1 and P2), GA+E2 (G1 and Q2), TR+E2+11-KT (H1 and R2), MN+E2+11-KT (I1 and S2) and GA+E2+11-KT XY fish (X and H2) at 60 and 90 dah. dah, day after hatching. The positive signal corresponds to the brownish color. IC, interstitial cells; OC, oocyte; SC, Sertoli cell; SG, spermatogonia. Scale bar: 5 µm.

Figure 3

Gene expression in XX, XY control and XY treatment fish at 90 dah by real-time PCR. *foxl2* was significantly upregulated in the gonads of the E2, TR+E2, MN+E2 and GA+E2 XY fish at 90 dah, compared with that of the control XY and the other XY treatment fish. The expression of *sf1* was significantly downregulated in gonads of the TR, MN, GA, E2, TR+E2, MN+E2 and GA+E2 XY fish, compared with that of 11-KT rescue and the control XY fish (A). The expression of *cyp19a1a* displayed similar pattern to that of *foxl2*. The expression of *cyp11b2* was significant downregulated in gonads of all treatment XY fish, compared with that of the control XY fish (B). Data were expressed as the mean $\pm$ s.d. ($n=6$). The expression level was normalized using the geometric mean of the levels of three internal control genes (*β -actin*, *gapdh* and *eEF1A1a*). Different letters above the error bar indicate significant difference ($P < 0.05$) as determined by one-way ANOVA followed by Duncan *post hoc* test.

testicular tissue with spermatogonia was observed at 60 dah, 1–3 oocytes were observed adjacent to the blood vessel in the ovotestis at 90 dah, 9–23 oocytes appeared in the central area of the gonad at 120 dah and only ovarian tissue was observed at 180 dah (Fig. 1I, J, K and L). The E2 SSR fish displayed delayed male-to-female sex reversal compared with the GA+E2 SSR fish, with testicular tissue observed at 60, 90 and 120 dah, ovotestis with ovarian tissue near blood vessel and testicular tissue opposite to blood vessel observed at 180 dah (Fig. 1M, N, O and P).

Figure 4

Serum E2 (A) and 11-KT (B) level of XX, XY control and XY treatment fish at 90 dah by enzyme immunoassay. The serum E2 level was significantly upregulated in E2, TR+E2, MN+E2, GA+E2, TE+E2+11-KT, MN+E2+11-KT and GA+E2+11-KT XY fish, compared with that of control XY, TR, MN and GA XY fish at 90 dah (A). The serum 11-KT level was significantly upregulated in TE+E2+11-KT, MN+E2+11-KT and GA+E2+11-KT XY fish, but significantly downregulated in E2, TR, MN, GA, TR+E2, MN+E2 and GA+E2 treatment XY fish at 90 dah, compared with that of control XY fish (B). Data were expressed as the mean $\pm$ s.d. ($n=6$). Different letters above the error bar indicate significant difference ($P < 0.05$) as determined by one-way ANOVA followed by Duncan *post hoc* test.

Gene expression during SSR in XY fish treated from 30 to 90 dah

The TR+E2, MN+E2 and GA+E2 SSR fish were used for gene expression profile and serum hormone level analyses. By IHC, Cyp19a1a was found to be expressed in the interstitial cells of the ovary in the control XX

Figure 5

Expression of Cyp19a1a, Cyp11b2 and Dmrt1 in gonad of XX, XY control and XY treatment fish at 120 dah by immunohistochemistry. The XY fish were treated by E2 alone (150 µg/g diet) or in combination with TR, MN and GA (150 µg/g diet) from 60 to 120 dah, and then fed with normal commercial diet till 180 dah. The control fish were raised in steroid-free water and fed with normal commercial diet. Cyp19a1a (A, B, C, D, E and F) was found to be expressed in the interstitial cells of the control XX (A). Cyp11b2 (G, H, I, J, K and L) was found to be expressed in the Leydig cells of the control XY, E2, TR + E2, MN + E2 and GA + E2 gonads at 120 dah (H, I, J, K and L). Dmrt1 (M, N, O, P, Q and R) was found to be expressed in Sertoli cells of the control XY, E2, TR + E2, MN + E2 and GA + E2 gonads at 120 dah (N, O, P, Q and R). The positive signal corresponds to the brownish color. IC, interstitial cells; OC, oocytes; PSC, primary spermatocytes; SC, Sertoli cells; SG, spermatogonia; SSC, secondary spermatocytes. Scale bar: 10 µm.

fish, but not in the testis of the control XY, TR, MN and GA treatment XY fish at 60 dah (Fig. 2A, B, C, D, E, F, G, H, I, J, K and L). Cyp11b2 and Dmrt1 were found to be expressed in the Leydig and Sertoli cells of the testis, respectively, in the control XY, TR, MN and GA treatment XY fish (Fig. 2N, O, P, Q, R, S, T, U, V, W, X and Z, A1, B1, C1, D1, E1, F1, G1, H1, I1, J1), but not in the ovary of the control XX fish (Fig. 2M and Y). Interestingly, Cyp19a1a was found to be expressed in the ovary of the control XX fish and the ovotestis of the TR+E2, MN+E2 and GA+E2 SSR fish (Fig. 2K1 and Q1, R1, S1), but not in the testis of the control XY, TR, MN, GA, E2, TR+E2+11-KT, MN+E2+11-KT and GA+E2+11-KT XY fish (Fig. 2L1, M1, N1, O1, P1 and T1, U1, V1). Cyp11b2 and Dmrt1 were found to be expressed in the ovotestis of the TR+E2, MN+E2, GA+E2 SSR fish and in the testis of the control XY, TR, MN, GA, E2, TR+E2+11-KT, MN+E2+11-KT and GA+E2+11-KT XY fish (Fig. 2X1, Y1, Z1, A2, B2, C2, D2, E2, F2, G2, H2 and J2, K2, L2, M2, N2, O2, P2, Q2, R2, S2, T2), but not in the ovary of the control XX fish (Fig. 2W1 and I2). Later, Cyp19a1a was detected in the ovary of the SSR XY fish at 180 dah, whereas Cyp11b2 and Dmrt1 were disappeared. Unexpectedly, Cyp19a1a, Cyp11b2 and Dmrt1 were found to be expressed simultaneously in the ovotestis of E2 XY fish (Supplementary Fig. 1).

Consistently, the expression of *foxl2* in the gonad of the TR+E2, MN+E2 and GA+E2 SSR fish was significantly higher than that of the E2 XY, control XY, TR, MN, GA, TR+E2+11-KT, MN+E2+11-KT and GA+E2+11-KT XY fish, which displayed no significant difference among themselves, except E2 XY fish, by real-time PCR. In contrast, the expression of *sf1* in TR+E2, MN+E2 and GA+E2 SSR fish was significantly lower than that of TR, MN, GA, E2 XY fish, which was significantly lower than that of the 11-KT rescued and control XY fish (Fig. 3A). However, the expression of *dmrt1* and *gsdf* in the gonad of the E2, TR, MN and GA XY fish was significantly higher than that of the TR+E2, MN+E2, GA+E2, TR+E2+11-KT, MN+E2+11-KT, GA+E2+11-KY and control XY fish, which showed no significant differences among themselves (Supplementary Fig. 2A). The expression of *cyp19a1a* displayed similar pattern to that of *foxl2*. The expression of *cyp11b2* displayed similar pattern to that of *sf1*, except the 11-KT rescued XY fish, which displayed significantly lower level than that of the control XY fish (Fig. 3B). The expression of *cyp26a1* was significantly upregulated, whereas the expression of *scp3* was significantly downregulated, in the gonad of the E2, TR, MN and GA XY fish, compared with that of the control XY fish (Supplementary Fig. 2B).

Figure 6

Serum E2 (A) and 11-KT (B) level in XX, XY control and XY treatment fish at 120 dah by enzyme immunoassay. The serum E2 level was significantly upregulated in the E2, TR+E2, MN+E2 and GA+E2 XY fish, compared with that of control XX and XY fish, whereas the serum 11-KT level was significantly downregulated in the E2, TR+E2, MN+E2 and GA+E2 XY fish, compared with that of the control XY fish. Data were expressed as the mean $\pm$ s.d. ($n=6$). Different letters above the error bar indicate significant difference ($P<0.05$) as determined by one-way ANOVA followed by Duncan *post hoc* test.

Serum E2 and 11-KT levels of XY fish treated from 30 to 90 dah

EIA analysis showed that the serum E2 levels of E2, TR+E2, MN+E2, GA+E2, TR+E2+11-KT, MN+E2+11-KT and

Figure 7

Expression of Cyp19a1a, Cyp11b2 and Dmrt1 in gonads of XX, XY control and XX treatment fish at 30 dah by immunohistochemistry. The XX fish were exposed to A4, testosterone and DHT (50 µg/L water) from 0 to 5 dah, and then fed with A4, testosterone and DHT (50 µg/g diet) until 30 dah. The control fish were raised in steroid-free water and fed with normal commercial diet. Cyp19a1a was only found to be expressed in the control XX gonad (A, B, C, D and E). Cyp11b2 and Dmrt1 were found to be expressed in the control XY and A4, testosterone, DHT XX gonads (F, G, H, I, J and K, L, M, N, O). The positive signal corresponds to the brownish color. IC, interstitial cells; OC, oocytes; SC, Sertoli cells; SG, spermatogonia. Scale bar: 5 µm.

GA+E2+11-KT XY fish were 140–180 times higher than those of the control XX fish, whereas the control XY, TR, MN and GA XY fish showed no significant difference among themselves at 90 dah (Fig. 4A). In contrast, the serum 11-KT levels of the TR+E2+11-KT, GA+E2+11-KT and MN+E2+11-KT XY were 140–160 times higher than those of the control XY, TR, MN, GA, E2, TR+E2, MN+E2 and GA+E2 XY fish at 90 dah. The serum 11-KT levels of TR, MN, GA and E2 XY fish were significantly lower than those of the control XY fish.

The serum 11-KT levels of TR+E2, MN+E2 and GA+E2 SSR fish were significantly lower than those of control XY and other treatment XY fish, except GA XY fish, at 90 dah (Fig. 4B).

SSR rate, gene expression and serum hormone levels of XY fish treated from 60 to 120 dah

When the gonad phenotype was examined histologically at 180 dah, all control XY fish developed as male with

normal testis and all control XX fish developed as female with normal ovary. Treatment of XY fish with E2 alone or in combination with MN and GA resulted in no male-to-female SSR, whereas with TR+E2, resulted in 3.3% SSR (Supplementary Table 3).

By IHC, Cyp19a1a was found to be expressed in the gonad of the control XX fish at 120 dah, and the control XX, TR+E2 XY SSR fish at 150 dah, but not in the gonad of the control XY, E2, TR+E2, MN+E2 and GA+E2 XY fish (Fig. 5A, B, C, D, E, F and Supplementary Fig. 3A, B, C, D, E, F). Cyp11b2 and Dmrt1 were found to be expressed in the gonad of the control XY, E2, TR+E2, MN+E2 and GA+E2 XY fish, but not in the gonad of control XX fish, at 120 and 150 dah (Fig. 5G, H, I, J, K, L, M, N, O, P, Q, R and Supplementary Fig. 3H, I, J, K, L, M, N, O, P, Q, R).

EIA analysis showed that the serum E2 level of the E2, GA+E2, MN+E2 and TR+E2 XY fish was 90 times higher than that of the control XX fish at 120 dah. The serum 11-KT level of the E2, GA+E2, MN+E2 and TR+E2 XY fish was significantly lower than that of the control XY fish, but significantly higher than that of the control XX fish (Fig. 6A and B). Although at 150 dah, 30 days after the withdrawal of the exogenous E2, the serum E2 of XY treatment fish dropped to a level lower than that of control XX fish, and the serum 11-KT level was still lower than that of control XY fish (Supplementary Fig. 3S and T).

Treatment of XX fish by A4, testosterone and DHT from 5 to 30 dah resulted in PSR

PSR of XX fish was successfully induced by A4 (sex reversal rate, 2/10), testosterone (9/10) and DHT (8/10) treatment. The sex reversed XX fish were used for gene expression profile analysis. By IHC, Cyp19a1a was found to be expressed in the gonad of the control XX fish, but not in the control XY, A4, testosterone and DHT XX fish at 30 dah (Fig. 7A, B, C, D and E). Cyp11b2 and Dmrt1 were found to be expressed in the gonad of the control XY, A4, testosterone and DHT XX fish, but not in the control XX fish (Fig. 7F, G, H, I, J and K, L, M, N, O).

Discussion

In fish, estrogens are synthesized during the key time of sex differentiation in females and play a critical role in sex differentiation and maintenance. In contrast, whether androgens are synthesized in the undifferentiated gonad to mediate sex differentiation in male is still controversial.

In medaka, tilapia and some other fishes, Cyp11b2 and 11 β -HSD2 are not detected before the morphological differentiation of the testis (Nakamura *et al.* 1998, Ijiri *et al.* 2008). However, in pejerrey, *cyp11b1* and 11 β -HSD2 are expressed, and androgens are synthesized in male undifferentiated gonads (Hattori *et al.* 2009, Blasco *et al.* 2010, 2013, Fernandino *et al.* 2012, 2013). In both cases, once the gonadal morphological differentiation has been started, Cyp11b1/2, 11 β -HSD2 and androgens are significantly upregulated (Nakamura *et al.* 1998, Vizziano *et al.* 2007, Blasco *et al.* 2010). Male-to-female sex reversal is restricted to morphologically undifferentiated stage by administration of exogenous estrogens (Guiguen *et al.* 1999, Kobayashi & Iwamatsu 2005, Chen *et al.* 2016). This led us to hypothesize that androgens antagonize the exogenous estrogens-induced male-to-female SSR after gonadal morphological differentiation.

Androgens are essential for male sex maintenance and antagonize E2-induced male-to-female sex reversal

In mammals, TR, MN and GA are the inhibitors of 3 β -HSD, Cyp11b2 and 11 β -HSD, respectively (Marandici & Monder 1993, Villeneuve *et al.* 2008, Rigel *et al.* 2010). In this study, TR, MN and GA treatment alone from 30 to 90 dah resulted in no sex reversal in XY fish as demonstrated by gonadal histology and the absence of Cyp19a1a expression at all time points checked. Consistently, no significant differences in the gonadal expression of *foxl2* and *cyp19a1a* mRNA and serum E2 level were detected compared with control XY fish. As expected, a significant decrease of *cyp11b2* mRNA and serum 11-KT level were detected in TR, MN and GA XY fish even though no sex reversal was observed, indicating that downregulation of endogenous androgen level alone could not induce male-to-female SSR. In contrast, treatment with E2 alone from 30 to 90 dah resulted in 8.3% male-to-female SSR in XY fish even though the fish displayed similar serum 11-KT level and *cyp11b2* expression to that of the TR, MN and GA XY fish. These results indicated that E2 has the same potential to inhibit androgen production as TR, MN and GA. Moreover, E2 has the ability to promote ovarian differentiation (Guiguen *et al.* 1999, Nagahama 2000), which was not possessed by TR, MN and GA. Interestingly, when TR, MN and GA were combined with E2, much higher rate of SSR was induced compared with that of the E2 XY fish. Further downregulation of serum 11-KT levels and *cyp11b2* expression, compared with that of the E2 XY fish, were detected in TR+E2, MN+E2 and GA+E2 SSR fish,

indicating double suppression of E2 and these inhibitors to androgen production. The sex reversal and gonadal gene expression were rescued by the administration of exogenous 11-KT. No differences in serum E2 level were observed for E2, TR+E2, MN+E2 and GA+E2 treatment fish at the end of the treatment. However, very low sex reversal rate and much longer sex reversal time were observed in E2 treatment fish compared with TR+E2, MN+E2 and GA+E2 treatment fish. This might be attributed to the significantly higher level of serum 11-KT in E2 XY fish compared with the other three treatment groups. These results indicated that E2 was essential for inducing male-to-female sex reversal, as demonstrated in the previous studies (Guiguen *et al.* 1999, Nagahama 2000, Kobayashi *et al.* 2003, Nakamura *et al.* 2003, Lee *et al.* 2004, Wu *et al.* 2008). Androgens antagonized exogenous E2-induced sex reversal of the XY fish with differentiated testis.

Treatment of XY fish with E2, TR+E2, MN+E2 and GA+E2 from 60 to 120 dah resulted in no male-to-female SSR, even though the serum E2 level of the treatment fish was significantly higher than that of the control XX fish and the serum 11-KT level was significantly lower than that of control XY fish at 120 dah. The androgen level might be not low enough to allow E2 to induce sex reversal in these XY fish. Only 3.3% of the TR+E2 XY fish displayed SSR at 150 dah. These results indicated that it becomes more and more difficult to suppress the gradually increased androgen production so as to induce male-to-female SSR during testicular development. Taken together, our results highlighted the critical roles of endogenous androgens in male sex maintenance.

Our hypothesis is also supported by the study in protandrous black porgy, in which the digonic gonad functions as testis for the first 2 years of life and male-to-female natural sex change occurs in the 3rd year (Wu *et al.* 2008). Male-to-female sex change could be induced by exogenous E2 treatment, but could not be maintained after the withdraw of exogenous E2; however, it could be achieved by surgical removal of the testis, in the younger than 2 years age fish (Lee *et al.* 2004, Wu *et al.* 2008, 2012, 2016). We suspect that androgens produced in the testicular tissue might be one of the anti-sex-change factors in this hermaphroditic species, as we proved in the gonochoric tilapia with differentiated testis.

Our previous study demonstrates that there is no significant differences in the PSR rate in XY fish treated from 0 to 30 dah by E2 alone (63%) or in combination with MT (52%), indicating that E2-induced PSR could not

be rescued by simultaneous administration of exogenous androgen (Chen *et al.* 2016). However, in the present study, the SSR induced by TR+E2, MN+E2 and GA+E2 in XY fish was successfully rescued by simultaneous administration of exogenous androgen 11-KT or MT. This difference could be explained by the fact that the androgen receptors (*ar1* and *ar2*) are expressed at relatively low levels, almost undetectable until approximately 15 dah in the gonad of the XY fish (Iriji *et al.* 2008, Tao *et al.* 2013, Cheng *et al.* 2015), and therefore, in the PSR fish, there is no receptor to mediate the androgen action during the key period of sex differentiation (5–10 dah), whereas in the SSR fish, androgens bind to the expressed receptors to antagonize E2-induced sex reversal.

Possible mechanism of androgens in male sex maintenance

In this study, significant up-regulation of *foxl2*, *cyp19a1a* was detected in the gonads of the E2, TR+E2, MN+E2 and GA+E2 SSR XY fish at 90 dah, compared with that of control XY fish. After the withdrawal of exogenous E2, *Cyp19a1a* expression was maintained and even upregulated in the gonads, which in turn, contributed to the higher serum E2 level of the SSR fish at 180 dah, compared with that of the control XY fish (Supplementary Fig. 4). These results indicated that exogenous E2-induced male-to-female sex reversal through increase of endogenous *Cyp19a1a* expression and estrogen production, as demonstrated in the previous studies (D'Cotta *et al.* 2001, Ijiri *et al.* 2008, Guiguen *et al.* 2010, Pérez *et al.* 2012, Chen *et al.* 2016, Wu *et al.* 2016). However, the upregulation of *foxl2* and *cyp19a1a* expression was rescued by administration of 11-KT in the XY treatment fish (Bhandari *et al.* 2006, Golan & Levavi-Sivan 2014), further demonstrated that androgens inhibited *foxl2* and *cyp19a1a* expression to antagonize exogenous E2 induction of SSR in XY fish. On the other hand, the downregulation of *sf1* expression could be attributed to the upregulated *foxl2* expression in the gonads of the TR+E2, MN+E2 and GA+E2 SSR fish at 90 dah. Transcriptional repression of *Foxl2* on *sf1* expression was proved in mouse and by our group in tilapia (Kashimada *et al.* 2011, Takasawa *et al.* 2014, Xie *et al.* 2016). However, expression of *dmrt1* and *gsdf* was not concomitantly downregulated in the gonads of the TR+E2, MN+E2 and GA+E2 SSR fish at 90 dah, but was downregulated to undetectable level at 180 dah, indicating that estrogen treatment upregulates *foxl2* and *cyp19a1a*, but does not suppress early testicular markers during male-to-female gonadal transdifferentiation as

Figure 8

Schematic representation showing experimental design, results and conclusion. Experiment 1: Inducement of SSR of 30 dah XY fish by androgen inhibitor and/or E2. The results demonstrated that androgens antagonize E2-induced male-to-female SSR. The upper the enzyme located in the steroidogenic pathway, the higher SSR rate was induced with E2 when it was inhibited. The SSR was successfully rescued by 11-KT. Experiment 2: Inducement of SSR of 60 dah XY fish by androgen inhibitor and/or E2. No SSR in E2, MN+E2 and GA+E2 XY fish, and only 3.3% SSR in TR+E2 XY fish were induced by androgen inhibitor and/or E2 treatment from 60 to 120 dah. The results demonstrated that it was very difficult or even impossible to induce SSR of more differentiated XY fish when androgen levels get higher. Experiment 3: Inducement of SSR in XX fish by exogenous A4, testosterone and DHT treatment. This explains why the upper the enzyme was located in the steroidogenic pathway, the higher SSR rate was achieved when it was inhibited by androgen inhibitor and induced by E2. Androgen precursors could function as androgens to antagonize E2-induced male-to-female sex reversal. Taken together, the results of these three experiment indicated that androgens are essential for male sex maintenance. Androgens antagonize E2 induced male-to-female sex reversal in differentiated testis. The lines and values colored red, blue and green indicate the treatment and induced SSR rate in experiments 1, 2 and 3, respectively.

demonstrated previously (Vizziano-Cantonnet *et al.* 2008). Taken together, we speculate that androgens antagonize E2-induced SSR of XY fish probably through direct inhibition of E2-induced female pathway gene expression.

The upper the enzyme located in the steroidogenic pathway, the higher SSR rate was induced with E2 when it was inhibited

A4 is an active androgen and substrate for biosynthesis of functional androgens (DeQuattro *et al.* 2015). Its product 11-hydroxyandrostenedione (11-OHA) induced sex reversal in the rainbow trout, by downregulation of Cyp19a1a (Govoroun *et al.* 2001). Testosterone could induce female-to-male sex reversal in medaka (Iwamatsu *et al.* 2006). DHT increased the levels of 11-KT and decreased the E2/11-KT ratio in both sexes in rainbow fish (Bhatia & Kumar 2016). In amphibians, female-to-male sex reversal was induced by high concentration of testosterone and DHT (Okada *et al.* 2008, Phuge &

Gramapurohit 2014, Xu *et al.* 2015). In the present study, female-to-male PSR was successfully induced by treatment of XX tilapia from 0 to 30 dah with A4, testosterone and DHT alone. The expression of Dmrt1 and Cyp11b2 was detected and Cyp19a1a was not detected in the gonads of A4, testosterone and DHT treatment fish at 30 dah. These results demonstrate that these androgen precursors could act as androgen in fish.

In this study, male-to-female SSR rate of the TR+E2 XY fish (88.3%) was higher than that of the MN+E2 (60%) and the GA+E2 XY fish (46.7%). The inhibition of 3β-HSD with TR resulted in decreased levels of A4 and testosterone in catfish, the precursors for synthesis of DHT, 11β-hydroxytestosterone and 11-KT in males (Mishra & Chaube 2017). In our TR+E2 XY fish, by inhibition of 3β-HSD, the synthesis of A4, testosterone, DHT, 11β-hydroxytestosterone and 11-KT was reduced, which significantly attenuated the antagonistic effects on E2-induced SSR. In MN+E2 XY fish, by inhibition of Cyp11b2, the synthesis of 11β-hydroxytestosterone and 11-KT was reduced, which attenuated the antagonistic effects

of androgens on E2-induced sex reversal to a moderate degree and resulted in intermediate SSR rate. In GA+E2 XY fish, by the inhibition of 11 β -HSD, the synthesis of 11-KT, but not A4, testosterone, DHT and 11 β -hydroxytestosterone, was reduced, which weakly attenuated the antagonistic effect of androgens on E2-induced sex reversal and resulted in the lowest SSR rate. That is, the upper the enzyme is located in the steroidogenic pathway, the higher SSR rate was induced with E2 when it was inhibited.

Orientation of SSR in XY gonad

In tilapia, the steroidogenic cells (aromatase positive) are firstly observed in a restricted area near the blood vessel at 5 dah, and the ovarian cavity is formed ventral to the ovary proper, opposite to the blood vessel at around 35–50 dah. Similarly, oogenesis proceeds as cluster of oocytes from area adjacent to blood vessel to the opposite in ovary (Nakamura *et al.* 1989, Strüssmann & Nakamura 2002, Wang *et al.* 2007, Ruksana *et al.* 2011). These studies demonstrate that the differentiation of the bipotential gonad is from the dorsal side (near the blood vessel) to the ventral side in XX fish. In the present study, during male-to-female SSR in XY fish, the first appearance of Cyp19a1a-positive cells and oocytes were observed adjacent to the blood vessel in the ovotestis. The oocytes were gradually increased in numbers occupying the central area with Cyp19a1a-positive cells scattered as clusters of interstitial cells, and finally, the SSR gonad was completely filled with oocytes. These results indicated that transdifferentiation of the differentiated testis proceeds in the same direction like ovarian differentiation, initiated from the area near the blood vessel and ended at the opposite end of the XY gonad. This is different from the female-to-male SSR in XX tilapia, which is from the opposite end to the area near the blood vessel (Sun *et al.* 2014).

Delayed spermatogenesis in TR, MN and GA treatment XY gonad

It is well known that endogenous androgens play critical roles in spermatogenesis during testicular development (Billard 1982, Cavaco *et al.* 2001, Schulz & Miura 2002, Ruwanpura *et al.* 2010). In the present study, the serum 11-KT level of the E2, TR, MN and GA XY fish was significantly lower than that of the control XY fish. Consequently, spermatogenesis was delayed in those XY treatment fish. In mammals, *Dmrt1* allows Sertoli cells to participate in retinoic acid (RA) signal to prevent

meiosis and promote spermatogonia proliferation in the testis (Jørgensen *et al.* 2012). Loss of *Dmrt1* results in precocious meiosis in male mice (Matson *et al.* 2010). Our previous study in tilapia demonstrated that *cyp26a1*, the catabolic enzyme for RA, is critical for the meiotic initiation of germ cells. Inhibition or knockdown of *cyp26a1* in XY fish resulted in the upregulation of RA level and earlier initiation of meiosis and spermatogenesis, as demonstrated by upregulation of *scp3* (Feng *et al.* 2015). In the present study, significant upregulation of *dmrt1*, *cyp26a1* and downregulation of *scp3* were detected in the gonads of E2, TR, MN and GA XY fish, compared with that of control XY fish. Taken together, we concluded that the upregulation of *dmrt1* might be responsible for the delayed spermatogenesis in the treatment XY fish.

In summary, our study suggests that androgens are critical for male sex maintenance in teleosts. Androgens antagonize E2-induced male-to-female sex reversal in differentiated testis. Therefore, blockage of androgen synthesis and simultaneous administration of E2 can successfully induce the differentiated testis to transdifferentiate into functional ovary (Fig. 8). Our study provides a good model for studying the molecular mechanism of SSR in teleosts.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0551>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H S, T G and L S conceived and designed the experiments; H S, T G, Z L, L C and X J collected the sample; H S, T G and Z L performed the experiments;

H S and T G and L S performed the analyses; D W and H S wrote the paper and H S, T G, Z L and L S contributed equally to this work.

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