Transdifferentiation of Differentiated Ovary into Functional Testis by Long-Term Treatment of Aromatase Inhibitor in Nile Tilapia

Li-Na Sun, Xiao-Long Jiang, Qing-Ping Xie, Jing Yuan, Bao-Feng Huang, Wen-Jing Tao, Lin-Yan Zhou, Yoshitaka Nagahama, and De-Shou Wang

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education) (L.-N.S., X.-L.J., Q.-P.X., J.Y., B.-F.H., W.-J.T., L.-Y.Z., D.-S.W.), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, 400715 Chongqing, People's Republic of China; Solution-Oriented Research for Science and Technology (Y.N.) and Laboratory of Reproductive Biology (Y.N.), National Institute for Basic Biology, Okazaki 444–8585, Japan; and South Ehime Fisheries Research Center (Y.N.), Ehime University, Matsuyama 790–8577, Japan

Females with differentiated ovary of a gonochoristic fish, Nile tilapia, were masculinized by long-term treatment with an aromatase inhibitor (Fadrozole) in the present study. The reversed gonads developed into functional testes with fertile sperm. The longer the fish experienced sex differentiation, the longer treatment time was needed for successful sex reversal. Furthermore, Fadrozole-induced sex reversal, designated as secondary sex reversal (SSR), was successfully rescued by supplement of exogenous 17β-estradiol. Gonadal histology, immunohistochemistry, transcriptome, and serum steroid level were analyzed during SSR. The results indicated that spermatogonia were transformed from oogonia or germline stem cell-like cells distributed in germinal epithelium, whereas Leydig and Sertoli cells probably came from the interstitial cells and granulosa cells of the ovarian tissue, respectively. The transdifferentiation of somatic cells, as indicated by the appearance of doublesex- and Mab-3-related transcription factor 1 (pre-Sertoli cells) and cytochrome P450, family 11, subfamily B, polypeptide 2 (pre-Leydig cells)-positive cells in the ovary, provided microniche for the transdifferentiation of germ cells. Decrease of serum 17β-estradiol was detected earlier than increase of serum 11-ketotestosterone, indicating that decrease of estrogen was the cause, whereas increase of androgen was the consequence of SSR. The sex-reversed gonad displayed more similarity in morphology and histology with a testis, whereas the global gene expression profiles remained closer to the female control. Detailed analysis indicated that transdifferentiation was driven by suppression of female pathway genes and activation of male pathway genes. In short, SSR provides a good model for study of sex reversal in teleosts and for understanding of sex determination and differentiation in nonmammalian vertebrates. **(*Endocrinology* 155: 1476–1488, 2014)**

In vertebrates, sex of some species is determined either genetically, like in eutherian mammals (1), or environmentally, like in some reptiles (2). For most noneutherian vertebrates, sex is affected by both genetic and environmental factors. The latter include temperature, steroid hormone, and so on (3, 4). The undifferentiated gonad of noneutherian vertebrates can be induced to differentiate into either a testis or an ovary by steroid hormone treat-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received October 18, 2013. Accepted January 9, 2014.
First Published Online January 17, 2014

Abbreviations: AI, aromatase inhibitor; Cyp19a1a, cytochrome P450, family 19, subfamily A, polypeptide 1a; Cyp11b2, cytochrome P450, family 11, subfamily B, polypeptide 2; dah, day after hatching; dat, day after treatment; Dmrt1, doublesex- and Mab-3-related transcription factor 1; E2, 17β-estradiol; ERKO, estrogen receptor knockout; FCG, female control group; Foxl2, forkhead box l2; GSC, germline stem cell; IHC, immunohistochemistry; 11-KT, 11-ketotestosterone; MCG, male control group; NSR, natural sex reversal; PCNA, proliferating cell nuclear antigen; PSR, primary sex reversal; qPCR, quantitative real-time PCR; RG, rescued group; RNA-Seq, RNA Sequencing; RPKM, reads per kb per million reads; SPC, steroid-producing cell; SSR, secondary sex reversal; TG, Fadrozole-treated group; TUNEL, terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling.

ment, regardless of their genetic sex, which we named as primary sex reversal (PSR). Exogenous estrogen is one of the most important inducers of ovary differentiation in PSR. Genetic males can be reversed to phenotypic females with estrogens if the treatment is applied before sex differentiation in vertebrates, including marsupials (5), birds (6), reptiles (7), and teleosts (8–10). In species with natural sex reversal (NSR), increase of serum 17β-estradiol (E2) levels always associated with male to female sex reversal in protandrous fish (11, 12). In contrast, E2 levels have been found to decrease during female to male sex reversal in protogynous fish (13–16). These results indicate that estrogens are also critical for NSR in teleosts.

For gonochorists, it is well accepted that once sex is differentiated, functional sex reversal is unlikely to happen. Recently, transdifferentiation of granulosa cells to Sertoli cells was successfully achieved by estrogen receptor knockout (ERKO) and forkhead box l2 (Foxl2)KO (17, 18) and vice versa by doublesex- and Mab-3-related transcription factor 1 (Dmrt1)KO (19). A recent report even goes further by demonstrating that adult females of tilapia and medaka could be induced into functional male by Fadrozole (an aromatase inhibitor [AI]) treatment (20). However, transdifferentiation of ovary to testis and global gene expression profiles were unclear, especially for tilapia. Most of the studies on sex reversal at the molecular level performed thus far were focused on a very small number of well-characterized genes and mostly on NSR and PSR. A few analyses were performed on sex-related gene expression profile during PSR in some species using microarray (21–23). However, this approach can only provide limited gene information for the elucidation of sex reversal as well as sex determination and differentiation. In contrast, RNA Sequencing (RNA-Seq) technology can provide large-scale gene expression profiles of a given tissue from the genome level, as proved by our previous study (24).

The Nile tilapia (*Oreochromis niloticus*) is a gonochoristic fish with a XX/XY sex determination system. The morphological sex differentiation of tilapia takes place at around 23–26 days after hatching (dah) (25), whereas the molecular sex differentiation starts from around 5 dah. The availability of all genetic female (XX) and all genetic male (XY) fry, the short spawning cycle (14 d), and commercial value of all male population make it a good species for the study of sex determination and differentiation. In the present study, long-term treatment of 90-dah genetic XX tilapia with Fadrozole resulted in phenotypic male (we named as secondary sex reversal [SSR]). The sequential changes in gonad histology and serum estrogen and androgen levels were analyzed during SSR. Different treatment time was applied to fish of different age for successful sex reversal. The global gene expression profiles in gonads were analyzed during SSR by RNA-Seq. Our work provides a good model for the investigation of transdifferentiation of gonad and gene cascade governing sex reversal in fish and for the elucidation of molecular mechanism for sex determination and maintenance in nonmammalian vertebrates.

Materials and Methods

Animals and drug-induced sex reversal

Experimental fish were reared, and all-XX and all-XY progenies were obtained as described in our previous study (24). All-XX fish of 15, 30, 60, or 90 dah were divided into 2 groups, female control group (FCG) and Fadrozole-treated group (TG), respectively. Another Fadrozole-treated and E2-rescued group (RG) was added to the 90-dah XX fish. All-XY fish of the same age as FCG were set as male control group (MCG). TG fish were fed with a diet sprayed with 95% ethanol containing Fadrozole (Sigma) at a concentration of 200-μg/g diet, whereas FCG and MCG fish were fed with a diet sprayed with 95% ethanol only. RG fish were fed with a diet sprayed with 95% ethanol contain-

Table 1. Treatment Time Needed for Sex Reversal of Nile Tilapia Induced from Different Age

Treatment	Fish age (dah)	Genotype	Fish number	Treatment time (d)	Gonad phenotype	Survival rate (%)	The rate of sex reversal (%)
FCG		XX	130		ovary	80	0
MCG		XY	130		testis	86	0
TG	15	XX	50	15	testis	54	100
	30	XX	50	30	ovotestis	76	100
	60	XX	50	30	ovary/ovotestis	80	15
	60	XX	50	60	ovotestis	72	100
	90	XX	50	30	ovary	78	0
	90	XX	50	60	ovary/ovotestis	74	10.8
	90	XX	130	90	ovotestis	90	100
RG	90	XX	50	90	ovary	88	0

Fish age (dah) is the age of fish when treatment started. Gonad phenotype was determined by gonad histology at 180 dah. TG described in the text, if not specified, indicated 90-dah fish treated for 90 days.

ing Fadrozole (200-μg/g diet) and E2 (200-μg/g diet). All fish were reared in the same conditions except for food. Drug treatment time applied to the fry was based on their age (dah), and details are shown in Table 1. Treatment for RG fish was applied from 90 dah (0 d after treatment [dat]) to 180 dah (90 dat). Gonad phenotype was determined by gonad histology at 180 dah. All drug treatment experiments were repeated 3 times.

For treatment groups started with 90-dah-old fish, gonads and serum from FCG, TG, and MCG fish were sampled for histology and immunohistochemistry (IHC), RNA-Seq, quantitative real-time PCR (qPCR), and sex hormone assays.

All 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.

Gonadal histology, IHC, and terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling (TUNEL) staining

Gonads were dissected, fixed in Bouin's solution for 24 hours at room temperature, dehydrated, and embedded in paraffin. All tissue blocks were sectioned at 5 μm and stained with hematoxylin and eosin or used for IHC analysis. Gonads used in TUNEL staining were fixed in 4% paraformaldehyde.

Antibodies against cytochrome P450, family 19, subfamily A, polypeptide 1a (Cyp19a1a) (encoding aromatase, the key enzyme for E2 synthesis), cytochrome P450, family 11, subfamily B, polypeptide 2 (Cyp11b2) (encoding 11β-hydroxylase, the key enzyme for androgen 11-ketotestosterone [11-KT] synthesis), Dmrt1, and proliferating cell nuclear antigen (PCNA) were diluted 1:2500, 1:500, 1:100, and 1:1000 in use, respectively. IHC analyses were performed as described previously (26). Photographs were taken under Olympus BX51 light microscope (Olympus).

Apoptosis in oocytes was determined by TUNEL assay with In Situ Cell Death Detection kit, POD (Roche Molecular Biochemicals) following the manufacturer's instructions. The sections were counterstained with hematoxylin after TUNEL staining. Sections processed without terminal deoxynucleotidyl transferase were taken as negative control.

Serum E2 and 11-KT level assay

For treatment groups started with 90-dah-old fish, blood samples from FCG, MCG, and TG fish were collected from caudal vein at 0, 10, 20, 30, 45, 70, and 90 dat during treatment, kept at 4°C overnight. Sera were collected after centrifugation and stored at −20°C until use. Serum E2, 11-KT, and testosterone levels were measured using the EIA Assay kits (Cayman Chemical Co) following the manufacturer's instructions.

RNA-Seq, calculation of transcript expression levels, and identification of up- and down-regulated genes

RNA-Seq, calculation of transcript expression levels, and identification of up- and down-regulated genes were performed as previously described (24). Briefly, total RNAs were extracted from 45- and 90-dat TG fish using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was further treated with deoxyribonuclease 1 (ribonuclease free) to eliminate genomic DNA contamination. The oligo (dT) beads enriched mRNA was disrupted into short fragments (200–700 nt) using fragmentation buffer. These short fragments were used as templates for first- and second-strand cDNA synthesis using an Invitrogen cDNA synthesis kit (Invitrogen). A QiaQuick PCR purification kit (QIAGEN) was used to purify these fragments, and the elution buffer was used for end repair and addition of the poly (A) tail. Then, these short fragments were ligated with sequencing adapters. After agarose gel electrophoresis, fragments between 320 and 370 nt were cut from the gel for PCR amplification. Two cDNA libraries were constructed from the 2 samples and sequenced on an Illumina HiSeqTM 2000 instrument. Clean reads from each library were aligned to the reference genome (Orenil1.0) using Tophat with default parameters (27), and the reads per kb per million reads (RPKM) was calculated to reflect transcript abundance as reported previously (24, 28). The threshold of P value was determined by false discovery rate, which is widely set with 10^{-2} (29). False discovery rate of less than or equal to 10^{-2} and $\log_2$(XX_RPKM/XY_RPKM) $\geq$1 was used to identify up- and down-regulated genes during SSR. The transcriptomic data from 90- and 180-dah normal female (XX) and male (XY) gonads (24) were employed as initial and end female and male control (0- and 90-dat FCG, 0- and 90-dat MCG).

Quantitative real-time PCR

qPCR was carried out on a 7500 real-time PCR machine (Applied Biosystems) with SYBR Green I fluorescent label using β-actin as an internal control. Gonads of FCG, MCG, and TG were dissected at 0, 10, 20, 30, 45, 70, and 90 dat from the fish treated at 90 dah (0 dat). Total RNA was extracted from each sample and reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Six genes with clear sexual dimorphic expression pattern, including *cyp19a1a*, *cyp19a1b*, *cyp11b2*, *sf1*, *foxl2*, and *dmrt1* (for the symbols and full names of all genes mentioned in the text, see Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), were selected to validate the transcriptomic data by qPCR using gene-specific primers reported earlier (30), and primer sequences were listed in Supplemental Table 2. The relative abundance of 6 genes mRNA transcripts was evaluated using the formula: $R = 2^{-\triangle\triangle Ct}$, as described previously (31, 32).

Results

Female ovaries were converted into functional testes

When checking histologically at 180 dah, all differentiated ovaries of XX fish at the age of 15, 30, 60, and 90 dah were reversed to functional testes with fertile sperm after 15, 30, 60, and 90 days of Fadrozole treatment, respectively. However, for 60-dah XX fish treated for 30 days, and for 90-dah XX fish treated for 60 and 30 days, only 15%, 10.8%, and 0% of ovaries of treated fish were sex reversed, respectively (Table 1). The reversed gonads of TG fish were ovotestes with testicular tissue occupying the most area of the gonad (except for TG of 15-dah-old

fish). In contrast, all ovaries of RG fish remained unchanged, similar to those of FCG fish. Eggs from normal females were inseminated with sperms collected from the Fadrozole-treated fish, and the resulting progeny included only females.

The histology of FCG and MCG gonads from 0 to 90 dat in 90-dah-old fish was typical ovaries and testes, in which the former were mainly consisted of oocytes from perinucleolar to vitellogenic phase and the latter had produced sperms (Figure 1). TG gonads had no difference with FCG gonads except for apparent proliferation of germ cells at 10 dat (Figure 1, A, G, and M). From 20 to 45 dat, apparent proliferation of somatic cells was observed in TG gonads but not in FCG gonads. Degeneration of oocytes was observed in a process started with disintegrated nuclei, gradual shrunken of their cytoplasm. Small oocytes at perinucleolar stage degenerated earlier than those big previtellogenic or vitellogenic oocytes. A few oogonia or germline stem cell (GSC)-like cells were observed scattered in the germinal epithelium. At 45 dat, a group of synchronized meiotic germ cells, at the pachytene stage of spermatocytes, was observed near the ovary cavity (Figure 1, H–J and N–P). Until 70 dat, obvious testicular tissues appeared in TG gonads. Many oogonia or GSC-like cells distributed throughout the germinal epithelium around ovarian cavity. Some of them had transdifferentiated into spermatogonia, primary and secondary spermatocytes in cysts ventral to the ovarian cavity. Ovarian tissues were shrunken, occupying the dorsal portion of the gonad (Figure 1, K and Q). By 90 dat, TG gonad looked like a testis histologically. The testicular tissues composed of spermatogonia, primary and secondary spermatocytes, sperms and somatic cells, such as Sertoli cells and Leydig cells, displayed no differences with those of MCG. However, a few oocytes remained near the blood vessel (Figure 1, L, R, and X).

Furthermore, from cross-sections and longitudinal sections of the anterior, middle, and posterior part at 70- and 90-dat TG gonads, sex reversal was found to be a gradual process from posterior to anterior and from ventral to dorsal (Supplemental Figure 1).

Serum E2, 11-KT, and testosterone levels during SSR

During the treatment period, serum E2 level increased gradually from 0 to 90 dat for the FCG fish, whereas for the TG fish, it declined significantly from 10 dat and continuously dropped to a level similar to that of MCG at the end of treatment (Figure 2A). In contrast, the serum 11-KT level of the FCG fish was low and stable during the treatment period. No significant difference in 11-KT level was observed between TG and FCG before 30 dat. However, the TG 11-KT level increased continuously after 30 dat and reached the level slightly less than that of MCG at 90 dat (Figure 2B).

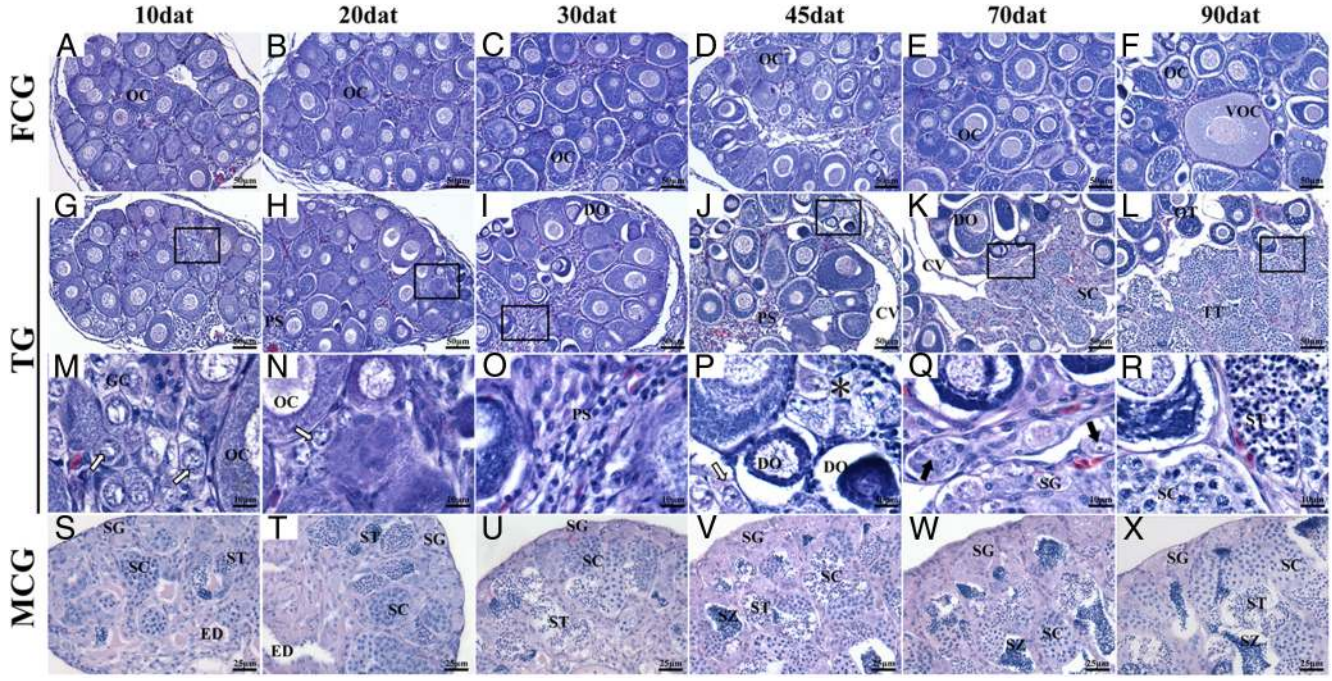

Figure 1. Histological observations of gonads from FCG, TG, and MCG fish during SSR. The treatment was applied to XX fish from 90 dah, lasted for 90 days, and resulted in SSR. A–F, FCG gonads; G–R, TG gonads. M–R, High magnification of the boxed areas in G–L, respectively. S–X, MCG gonads. White arrows in M, N, and P indicate oogonia; and asterisk in P indicates synchronized spermatocytes in a cyst. Black arrows in Q indicate transdifferentiated spermatogonia. OC, oocytes; VOC, vitellogenic oocytes; GC, germ cell; DO, degenerating oocytes; PS, proliferous somatic cell; CV, ovarian cavity; SG, spermatogonia; SC, spermatocytes; ST, spermatid; SZ, spermatozoa; ED, efferent duct; TT, testicular tissue; OT, ovarian tissue.

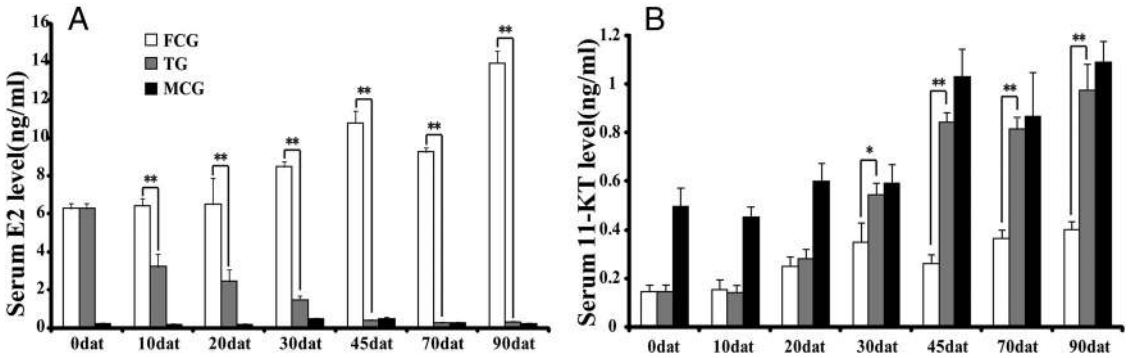

Figure 2. Serum E2 and 11-KT level in FCG, MCG, and TG during SSR by enzyme immunoassay. Data are expressed as the mean ± SD for 6 fish. * and ** represent significant difference at $P < .05$ and $P < .01$ between FCG and TG, respectively, by one-way ANOVA.

Serum testosterone levels increased continuously for both FCG and MCG fish, with FCG increased faster than MCG during the treatment period. In contrast, the serum testosterone levels of the TG fish increased rapidly from 0 to 30 dat (2- and 5-fold higher than that of FCG and MCG fish, respectively); then, dropped rapidly from 45 dat and kept to a level similar to that of MCG until the end of the treatment (Supplemental Figure 2).

RNA-Seq and read mapping

Sequencing of tilapia 45- and 90-dat TG gonadal transcriptomes yielded a total of 52 172 864 and 48 571 722 reads, respectively. The data have been deposited in NCBI's Short Read Archive at http://www.ncbi.nlm.nih.gov/sra/ under the accession number SRP014017. The clean reads from the 2 transcriptomes were mapped into 19 701 and 19 948 genes, respectively.

Gene counts, global gene expression profiles in gonad during SSR

The 2 TG transcriptomes were compared with the 4 normal gonadal transcriptomes (0- and 90-dat FCG and MCG) sequenced earlier by our group (24) in gene counts. The gene count of 45-dat TG (19 701) was larger than that of 0-dat FCG (18 203) but less than that of 90-dat TG (19 948) (Figure 3A), whereas the gene count of 90-dat TG was larger than that of 90-dat FCG (19 341) but less than that of 90-dat MCG (20 659).

To group the gonadal samples according to their global gene expression similarities, the gene expression profiles were analyzed with an unsupervised hierarchical clustering algorithm. The resulting dendrogram contained 2 main branches (I and II), with the 2 MCG samples clustered into branch I, whereas all TG and FCG samples clustered into branch II (Figure 3B).

Comparison of the gene expression level of TG and FCG gonads at 90 dat revealed that 754 genes were down-regulated, whereas 1591 genes up-regulated in TG gonads. The TG down- and up-regulated genes were compared with the XX- and XY-enhanced genes during normal developmental stages (24). Only 8 of 754 TG down-regulated genes were XX-enhanced genes (Figure 3C), whereas 306 of 1591 TG up-

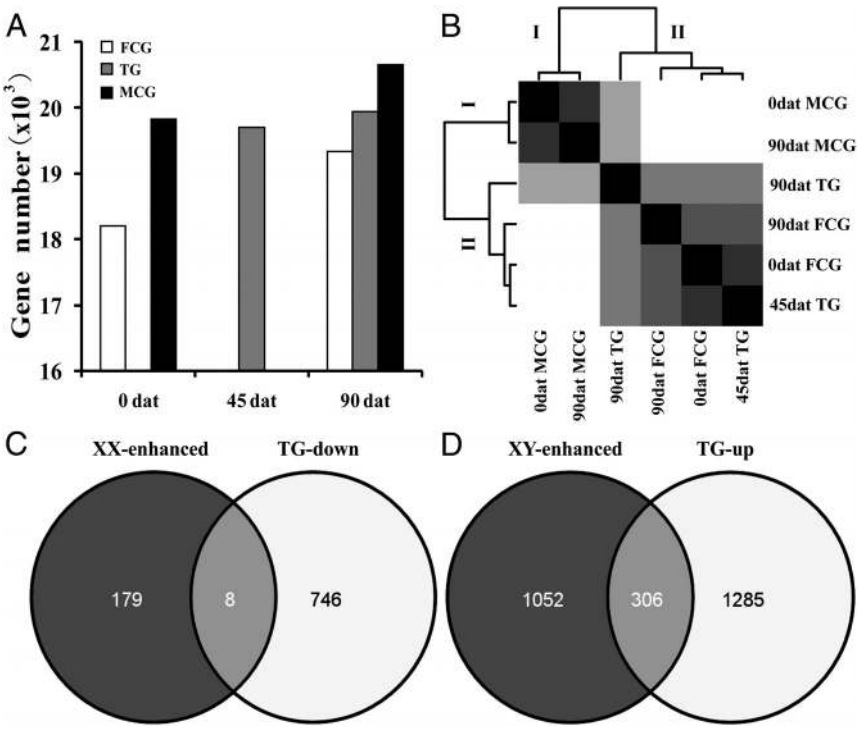

Figure 3. Transcriptomic analysis of gene expression profiles in gonad during SSR. A, Gene number of 0-, 45-, and 90-dat gonadal transcriptomes. B, Classification of 0-, 45-, and 90-dat gonadal transcriptomes using an unsupervised average linkage clustering of the gene expression profiles. C and D, Venn diagram of XX-enhanced Vs TG down-regulated genes (C) and XY-enhanced Vs TG up-regulated genes (D) during SSR.

regulated genes were XY-enhanced genes (Figure 3D). Detailed information (gene name and RPKM) of these 8 and 306 genes was shown in Supplemental Tables 3 and 4, respectively.

Expression profiles of steroidogenic enzyme genes in gonads during SSR

Comparison of the expression profiles of the steroidogenic enzyme genes, *star1*, *star2*, *cyp11a1*, *cyp17a1*, *cyp17a2*, *hsd3b1*, *cyp11b2*, *hsd11b2*, *cyp19a1a*, and *cyp19a1b* in TG with those in FCG and MCG revealed that most of them experienced an exponential increase in gonads during SSR (Figure 4). The average RPKM of the 10 steroidogenic enzymes rapidly increased from 33 at 0 dat to 85 at 45 dat and to 188 at 90 dat, whereas it remained almost unchanged from 33 at 0 dat to 35 at 90 dat in FCG and slightly increased from 379 at 0 dat to 470 at 90 dat in MCG. The expression levels of the steroidogenic enzymes in TG were much higher than those in FCG but still much lower than those in MCG at 90 dat. It is worth noting that the expression (RPKM) of *cyp19a1a* was increased significantly from 145 at 0 dat to 563 at 45 dat and then decreased dramatically to 122 at 90 dat in TG, less than that in FCG but much higher than that in MCG.

Expression profiles of key genes involved in sex differentiation during SSR by qPCR

From qPCR, the expressions of *cyp19a1a*, *cyp19a1b*, and *foxl2* were much higher in FCG than in MCG (Figure 5, A–C), whereas the expression levels of *cyp11b2*, *dmrt1*, and *sf1* were much higher in MCG than in FCG from 0 to 90 dat (Figure 5, D–F). *Cyp19a1b* and *foxl2* in TG gonads shared a similar expression patterns. Both of them decreased continuously during the masculinization process, slightly dropped at 10 dat, significantly reduced at 20 dat, and very significantly reduced from 30 to 90 dat, whereas *cyp19a1a* was increased at the early period of treatment (0–45 dat), declined rapidly from 70 dat, and finally became significantly lower than that in FCG at 90 dat. On the contrary, *sf1* started to increase from 10 dat, whereas *cyp11b2* and *dmrt1* from 30 dat, and became significantly higher in TG gonads than those in FCG at 90 dat. The expression profiles of these genes revealed by qPCR were consistent to those from our transcriptome data.

TUNEL staining and IHC

By TUNEL staining, positive signals were detected in the nuclei of oocytes in TG gonads, with stronger signals in the small oocytes (phase I), whereas no signals were observed in most the somatic cells except a few granulosa cells and interstitial cells, which displayed moderate positive signals (Figure 6B). In contrast, few positive signals were observed in sections of FCG (Figure 6A). By IHC of PCNA, strong positive signals were observed in proliferating somatic cells in TG gonad, whereas only dispersed positive signals were observed in FCG gonad (Figure 6, C and D).

Cyp19a1a was constantly expressed in the interstitial cells, some theca cells, and granulosa cells in FCG but never expressed in MCG (Figure 6, E and G, and Supplemental Figure 3), whereas Cyp11b2 expressed in Leydig cells in MCG but never expressed in FCG (Figure 6, F and H, and Supplemental Figure 4). In TG gonad, Cyp19a1a expressions were observed in the same cell types in ovarian tissue as observed in FCG but never in the testicular tissue (Figure 6, I, K, and M, and Supplemental Figure 3), whereas Cyp11b2 was expressed in Leydig cells in testicular tissue as observed in MCG (Figure 6, N and O, and Supplemental Figure 4). Consistent with the qPCR and transcriptomic data, Cyp19a1a-positive signals increased from 20 to 45 dat due to proliferation of steroidogenic

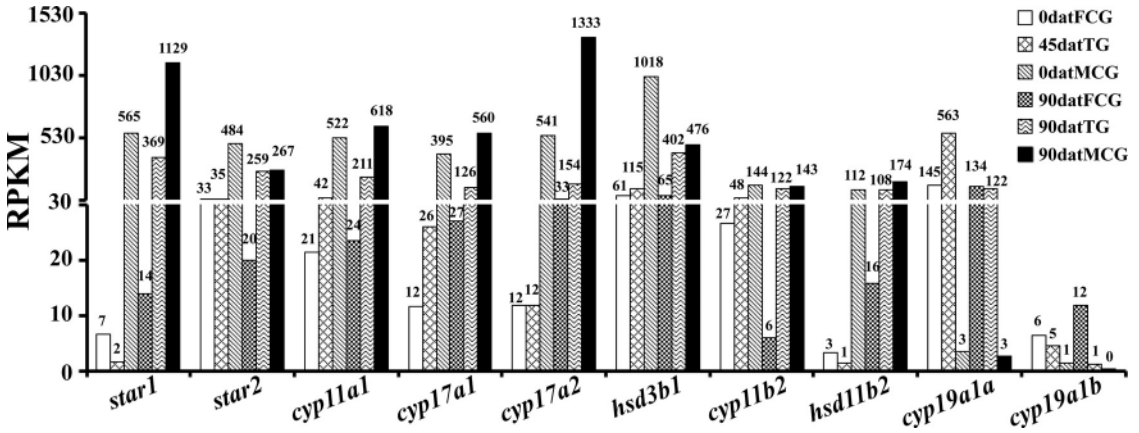

Figure 4. The expression profiles of steroidogenic enzyme genes during SSR. The expression levels of *star1*, *star2*, *cyp11a1*, *cyp17a1*, *cyp17a2*, *hsd3b1*, *cyp11b2*, and *hsd11b2* were up-regulated in TG during SSR, whereas the expression levels of *cyp19a1b* were down-regulated during SSR. In contrast, the expression of *cyp19a1a* was increased significantly from 0 to 45 dat and decreased dramatically from 45 to 90 dat.

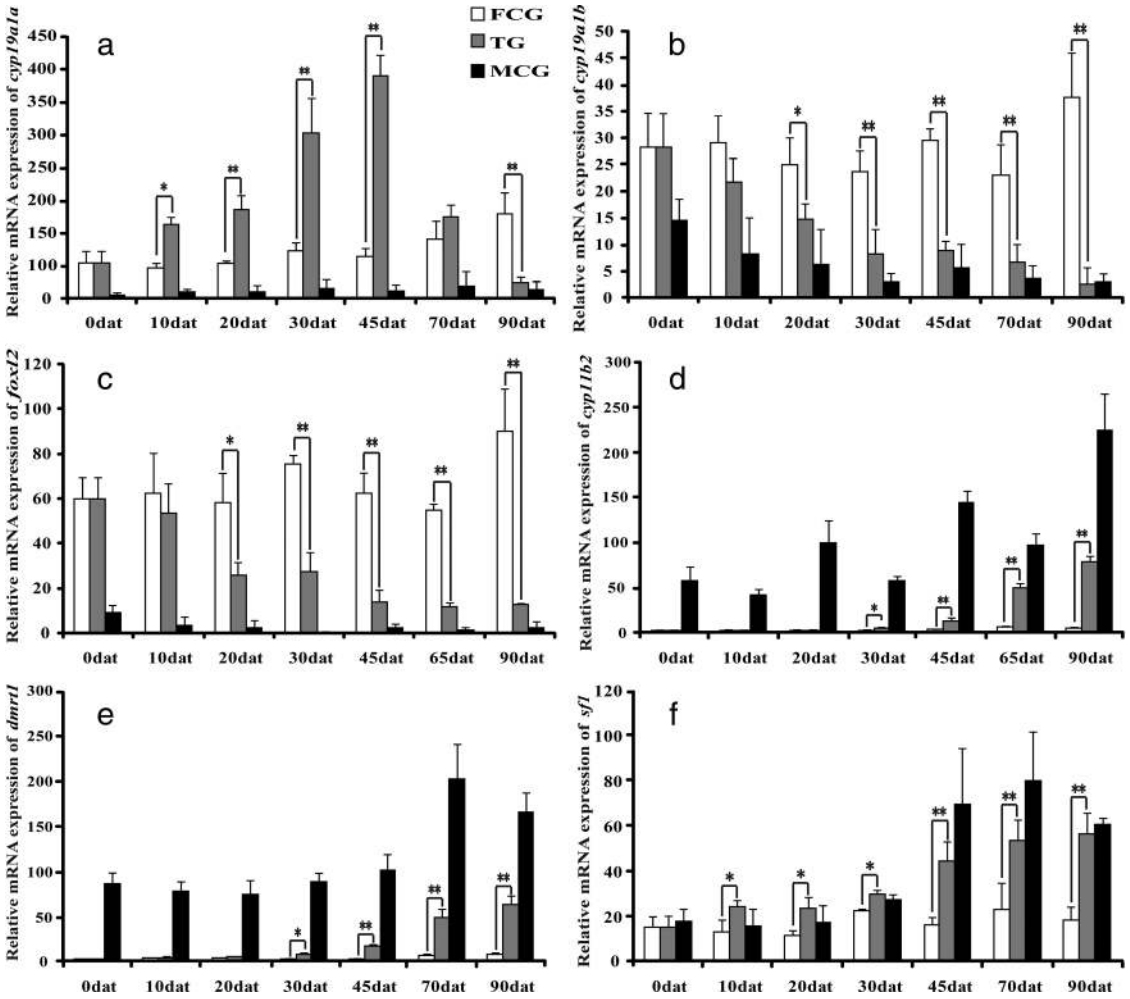

Figure 5. Expression profiles of 6 key genes known to be involved in sex differentiation during SSR analyzed by qPCR. The relative abundance of *cyp19a1a*, *cyp19a1b*, *foxl2*, *cyp11b2*, *dmrt1*, and *sf1* mRNA transcripts is evaluated using the formula: $R = 2^{-\Delta\Delta Ct}$. *β-Actin* was used as the internal control. Data are expressed as the mean ± SD for 3 replicates obtained from 9 different fish. * and ** represent significant difference at $P < .05$ and $P < .01$ between the TG and FCG gonads, respectively, by one-way ANOVA.

interstitial cells, then significantly decreased at 70 dat and almost disappeared at 90 dat except a few interstitial cells near the blood vessel and a few theca cells surrounding the remaining oocytes in the TG gonads (Supplemental Figure 3). To our surprise, Cyp11b2-positive signals, which were undetectable until 20 dat, were observed to be increased in those newly proliferated interstitial cells in the ovarian tissue of TG gonads from 30, 45, and 70 dat (Supplemental Figure 4). By IHC staining of adjacent sections in series from TG gonads, Cyp19a1a and Cyp11b2 were confirmed to be coexpressed in the same type of steroidogenic cells in ovarian tissue at 45 and 70 dat (Figure 6, I–L). With the degeneration of oocytes and the expansion of testicular tissue, these steroidogenic interstitial cells became Cyp11b2-positive (Figure 6, M and N) but no longer Cyp19a1a-positive (Figure 6, O and P).

Dmrt1-positive signals were observed in the Sertoli and epithelial cells comprising the efferent duct in MCG testis but not detected in FCG ovary at all time point examined (Figure 6, Q and R). Interestingly, Dmrt1-positive signals were also detected in somatic cells surrounding "oogonia" or GSC-like cells (or putatively known as spermatogonia), which scattered in germinal epithelium near the ovarian cavity at 30- and 45-dat TG gonads (Figure 6S and Supplemental Figure 5). In 70- and 90-dat TG, Dmrt1 signals were located in Sertoli cells surrounding spermatogonia or spermatogenic cysts in the testicular tissue and numerous spermatogonia distributed in germinal epithelium along both sides of ovarian cavity. Stronger positive signals were observed at cells surrounding the space left after oocyte degradation. In contrast, no Dmrt1 signal was detected in the ovarian tissue of the TG ovotestis (Figure 6T). In TG gonads, perinucleolar oocytes were surrounded by unilaminar granulosa cells, which were Dmrt1 negative in ovarian tissue (Figure 6U). When the oocyte degraded and disappeared, a cavity was left, and its surrounding granulosa cells remained and changed into Dmrt1-positive cells (Sertoli cells). Then, germ cells (spermatogonia) sur-

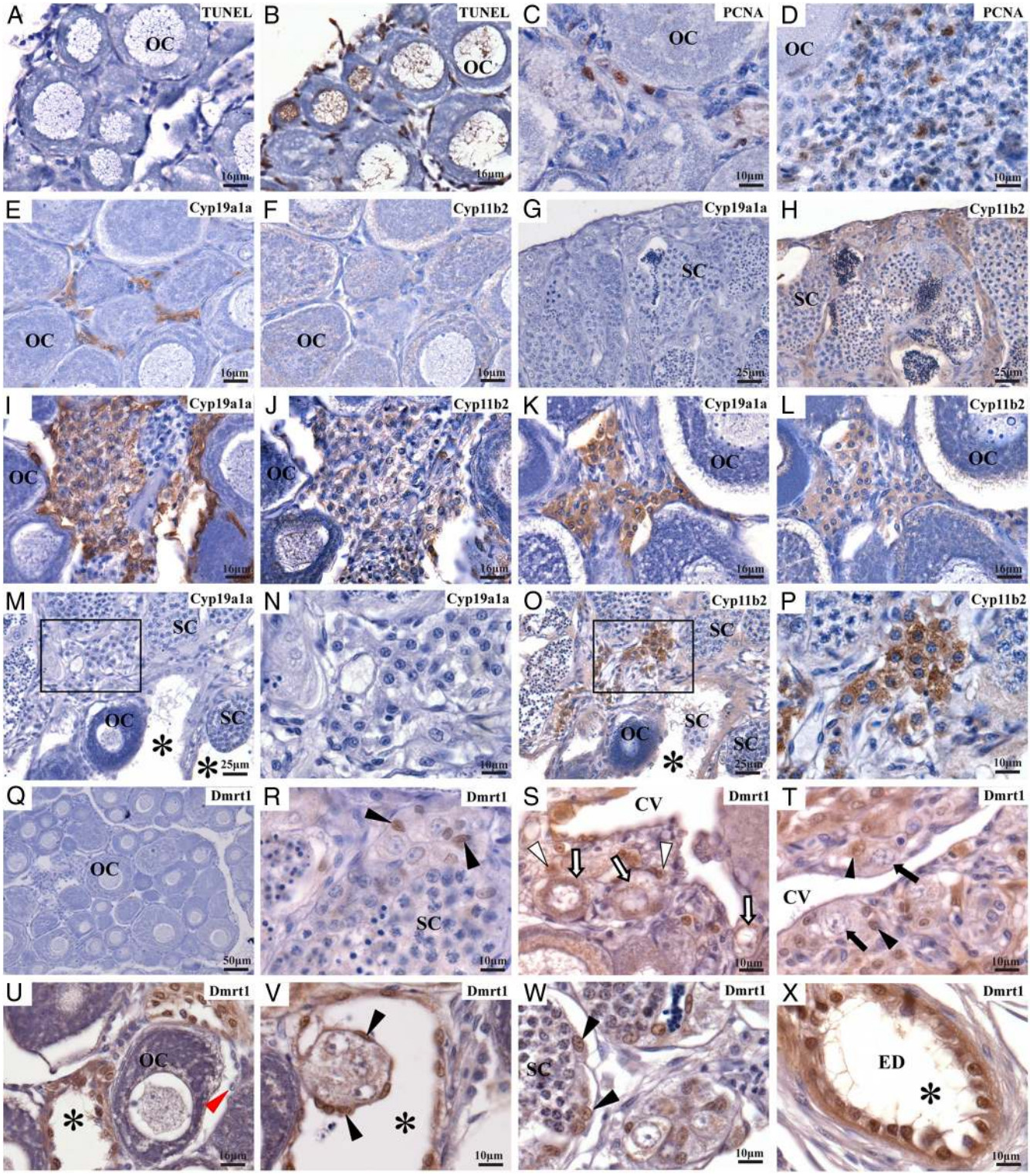

Figure 6. Transdifferentiation of germ cells and somatic cells of tilapia gonads by TUNEL staining (A and B) and IHC (C–X) during SSR. A and B, Apoptosis of oocytes and somatic cells by TUNEL staining of gonad from FCG (A) and TG (B) at 20 dat. C and D, Proliferation of somatic cells (steroidogenic cells) by IHC of PCNA from FCG (C) and TG (D) at 45 dat. E–P, Transdifferentiation of E2-producing cells to 11-KT-producing cells by IHC of Cyp19a1a and Cyp11b2 with adjacent sections of FCG (E and F), MCG (G and H) and TG (I–P). Cyp19a1a is detected in the interstitial and some theca cells in 0-dat FCG and TG (E), whereas Cyp11b2 is undetectable in same position (F). In contrast, strong Cyp11b2 signal was detected in Leydig cells in MCG (H), whereas Cyp19a1a was never detected in testicular tissue of MCG (G) and 90-dat TG (M). Coexpression of Cyp19a1a and Cyp11b2 in the steroid-producing interstitial cells was observed in the ovarian tissue at 45-dat (I and J) and 70-dat (K and L) TG. Once spermatogenic cells invaded the cavity left by degenerated oocytes, the steroidogenic interstitial cells became Cyp11b2-positive (O and P), but no longer Cyp19a1a-positive (M and N) at 90-dat TG gonad. N and P, Higher magnification of the boxed areas in M and O, respectively. Q–T, Transdifferentiation of oogonia to spermatogonia by Dmrt1 IHC. Dmrt1 was detected in Sertoli cells (black arrowheads) in MCG gonad (R) but

rounded by Dmrt1-positive Sertoli cells invaded the cavity (Figure 6V), and gradually, the cavity was filled with testicular tissue (Figure 6W). The Dmrt1-positive cells became epithelial cells comprising the efferent duct of testicular tissue if no germ cell were invaded in the space left by the degenerated oocytes (Figure 6X).

Discussion

Transdifferentiation of germ cell and somatic cell in gonad during SSR

Existing investigations on sex reversal were mostly focused on NSR or PSR in teleosts. In the NSR, artificial inducement was widely applied to hermaphroditic fish with differentiated ovary or testis to invert sexuality in advance by administration of exogenous steroids or AI (33–36). For gonochoristic fish, sex reversal of fish with undifferentiated bipotential gonads (PSR) could be achieved by similar administration before or during the narrow window of sex determination (9, 22, 23, 37, 38). Once the fish grows beyond the stage, artificially induced sex reversal is difficult or even impossible to accomplish. In this study, the differentiated genetic females (XX) of tilapia were successfully induced to phenotypic males with ovotestis being able to produce fertile sperm. To distinguish from NSR and PSR, we named it as SSR. Sex reversal was successfully induced with fish of different age. However, the treatment time needed for sex reversal was proportional to fish age (or the time of fish experienced sex differentiation), because older fish treated with shorter time resulted in no or very low rate of sex reversal. SSR is useful for study of sex reversal and for understanding of the molecular mechanism of sex determination and differentiation in teleosts as well as in other vertebrates.

During PSR, the undifferentiated gonad directly developed into the opposite phenotypic sex, and it was very difficult or even impossible to distinguish the sex-reversed gonad from normal testis or ovary histologically. The process of NSR and SSR experienced the degeneration of ovarian tissue and the reconstruction of testicular tissue, or vice versa. In most protogynous fish (39–41), except for saddleback wrasse (14), testicular tissues with a few developing cysts of male germ cells were observed dispersed in the ovarian tissue in the gonad. In protandrous black porgy, small ovarian tissue with a few primary oocytes dispersed in the testicular tissue was observed before sex reversal (33). Obviously, the new tissues came from the preformed testicular or ovarian tissue in the hermaphroditic fish during NSR. Where did germ cells and somatic cells of testicular tissue come from in the gonochoristic tilapia during SSR? Undifferentiated mitotic germ cells, designated as GSCs, were proved to be present in the germinal cradles in the ovary of adult medaka (42). In the present study, we observed that sex reversal was initiated from the germinal epithelium opposite to the blood vessel, ventral to the ovarian cavity, as demonstrated by the first appearance of Dmrt1-positive cells. In a previous study, dmrt1 was proved to express in Sertoli cells of testis, but not in ovary of Nile tilapia (43). Because there has been no report demonstrating the existence of GSCs in the differentiated ovary of tilapia up to now, the germ cells observed in the germinal epithelium were designated as oogonia or GSC-like cells, and the somatic cells were designated as germ cell-surrounding cells (Dmrt1-positive pre-Sertoli cells). At the early stage of SSR, oogonia or GSC-like cells and the surrounding somatic cells in the germinal epithelium were the major source of spermatogonia and pre-Sertoli cells as proved by Dmrt1 IHC. However, at the later stage, with the degeneration of many oocytes and the quick development of testicular tissue, migration of proliferated spermatogonia became the major source of germ cells, and transdifferentiation of granulosa cells became the important source of Sertoli cells. Dmrt1-positive cells also became epithelial cells comprising the efferent duct of testicular tissue if no germ cell were invaded in the space left by the degenerated oocytes. Our result is consistent with the evidence from ERKO, Foxl2KO mouse (17, 18) that ERKO or Foxl2KO in female mouse resulted in the transdifferentiation of granulosa into Sertoli-like cells.

Generally, Cyp19a1a (key enzyme for E2 synthesis) expresses in the steroid-producing cells (SPCs) in normal ovary but not in normal testis by IHC. However, Cyp11b2 (key enzyme for 11-KT synthesis) expresses in the Leydig cells in normal testis but not in normal ovary, ie, Cyp19a1a and Cyp11b2 were never found to be coexpressed in the normal ovary and testis by IHC. However, in the present study, IHC data demonstrated that prolif-

Figure 6 (Continued). never in FCG gonad (Q). Dmrt1 was detected in somatic cells (white arrowheads) surrounding the oogonia or GSC-like cells (white arrows) in germinal epithelium at 30 dat (S) and Sertoli cells (black arrowheads) at 70-dat (T) TG gonad, indicating the transdifferentiation of germ cells from oogonia to spermatogonia (black arrows). U–X, Transdifferentiation of granulosa cells to Sertoli cells and epithelial cells of the efferent duct by Dmrt1 IHC at 90-dat TG. Dmrt1 was not expressed in the granulosa cells (red arrowhead) surrounding oocytes (U), whereas it was expressed in the granulosa cells once the oocytes degenerated (V). The granulosa cells transdifferentiated to Sertoli cells (black arrowheads) if spematogonia invade the cavity left by the degenerated oocytes (V and W), otherwise they became epithelial cells of the efferent duct (X). Brown color, positive signals. Asterisks indicate the spaces left by degenerated oocytes. OC, oocyte; CV, ovarian cavity; SC, spermatocytes; ED, efferent duct.

erated somatic cells in TG gonad displayed strong positive signals of both Cyp19a1a and Cyp11b2. These data indicated that the proliferated somatic cells, if not all, were SPCs. More importantly, Cyp11b2 was detected in SPCs of the ovarian tissue in TG gonad, coexpressed with Cyp19a1a. When spermatogenic cysts invade the space left by degenerated oocytes, those cells were still Cyp11b2-positive but no longer Cyp19a1a-positive, suggesting that the Leydig cells of the testicular tissue from TG gonad were transdifferentiated from the interstitial SPCs of ovarian tissue. Furthermore, the change of steroidogenic enzymes resulted in the production of androgen 11-KT in these cells, which, in turn, provides the extracellular environment for spermatogenesis.

Transdifferentiation was driven by the suppression of female pathway genes and the activation of male pathway genes

According to our previous study on gonadal transcriptome of 4 developmental stages in Nile tilapia, male gonads always express more genes than female gonads, suggesting that differentiation and development of male gonads rely on more genes than female gonads (24). In the present study, increased number of genes was found to be expressed in 45- and 90-dat TG transcriptomes during SSR, which is consistent with the notion that more genes are required for the development of phenotypic male gonad. Furthermore, the gene number of 45- and 90-dat TG gonads is larger than that of 0- and 90-dat FCG gonads, indicating that the expression of some genes involved in male pathway are initiated during SSR. The 754 down-regulated genes in TG gonads were considered as genes related to female phenotypic sex differentiation and maintenance, whereas the 1591 up-regulated genes related to testicular phenotypic differentiation and maintenance. The identification and characterization of down- and up-regulated genes in TG gonads is of vital importance for us to understand how these genes involved in gonadal transdifferentiation.

However, the global expression profiles of TG gonads showed a better correlation with FCG gonads as classified using an unsupervised average linkage clustering of the gene expression profiles, indicating that the global expression profiles of FCG, MCG, and TG gonads were clearly discriminated by their genetically sex, ie, XY (MCG gonads)/XX (FCG and TG gonads). Similar results were also reported in masculinized XX rainbow trout (22). All these results indicated that although the phenotype of the gonads of 90-dat TG displayed more similarity in morphological and histological characteristics with MCG, the global gene expression profiles were closer to those of FCG. Therefore, the gonadal transdifferentiation was driven by the suppression of female pathway genes and the activation of male pathway genes, even though those down- and up-regulated genes were a small number of gonad-expressed genes.

Only 8 of 754 TG down-regulated genes were XX-enhanced genes, in which *cyp19a1b* (44–46), *wnt5a* (47), and *foxl2* (48–53) have been reported to be related to ovarian differentiation and development. However, more TG up-regulated genes (306 of 1591) were XY-enhanced genes, including *dmrt1* (30, 54–56), *foxJ1a* (57), and *cyp17a1* (58). The decrease in expression of these XX-enhanced genes and the increase in expression of these XY-enhanced genes during SSR suggested their crucial roles in sex reversal as well as in ovarian maintenance and in testicular development.

Down-regulation of endogenous estrogen is prerequisite for the ovarian transdifferentiation

It is well known that estrogens play a pivotal role in gonadal sexual differentiation in teleost (4, 59). Decrease of endogenous estrogen always associated with the sex reversal from female to male in PSR and NSR. In the present study, Fadrozole directly inhibited the activity of aromatase in the ovary, resulting in the continuous and sharply decrease of serum E2 levels from 10 to 30 dat. The decrease of E2 suppressed the expression of female pathway genes as revealed by transcriptome analysis. Especially, the 2 key genes, *foxl2* and *cyp19a1b*, were found significantly decreased from 20 dat by qPCR, indicating their critical role in gonadal transdifferentiation. Expression of *cyp19a1b* was continuously decreasing during SSR, parallel to the estrogen decrease, indicating that *cyp19a1b* may be also involved in gonadal estrogen synthesis and ovarian maintenance. *Cyp19a1a* was regarded as an important factor in ovary maintenance, and down-regulation of *cyp19a1a* is the only necessary step for inducing a testicular differentiation pathway in PSR and NSR (60). Unexpectedly, during Fadrozole treatment the expression of *cyp19ala* significantly increased to compensate for decreased aromatase activity and E2 levels in TG gonads from 10 to 45 dat, then significantly decreased from 70 to 90 dat when obvious testicular tissue appeared, which were demonstrated by transcriptome analysis, qPCR, and IHC. IHC results indicated that the Cyp19a1a up-regulation during 10 to 45 dat was caused by the proliferation of Cyp19a1a-positive cells, whereas its down-regulation from 70 to 90 dat was due to the transdifferentiation of Cyp19a1a-positive cells to Cyp11b2-positive cells. The expression profile of *cyp19a1a* in SSR is different from the continual decrease of *cyp19a1a* observed during PSR in rainbow trout (23), Japanese flounders (61) as well as tilapia (62), and NSR in European sea bass (63),

suggesting a remarkable difference between SSR with PSR and NSR. Our results indicated that down-regulation of Cyp19a1a activity, which resulted in decrease of E2, is the prerequisite for AI-induced gonadal transdifferentiation in SSR. This conclusion was further supported by the fact that supplying exogenous E2 with Fadrozole treatment successfully rescued the latter induced sex reversal.

Our data demonstrated that decrease of E2 (10 dat) was earlier than increase of 11-KT (30 dat) during SSR. Serum testosterone level in TG was significantly higher than that of FCG and MCG at 20 and 30 dat and sharply decreased from 45 dat to a level significantly lower than that of FCG but similar to that of MCG until the end of the treatment, which is negatively correlated to the level of Cyp11b2 and 11-KT during the same period. Transcriptome analyses also demonstrated an up-regulation of steroidogenic enzymes in gonads during SSR. These results indicated that the up-regulation of steroidogenic enzymes and the blockage of aromatase activity by AI resulted in the accumulation of serum testosterone levels in the early stage of treatment. After 45 dat, the up-regulation of Cyp11b2 resulted in increased conversion of testosterone to 11-KT and, therefore, responsible for the decrease of testosterone and increase of 11-KT. Our results indicated that E2 plays a key role in the ovary maintenance, and lack of E2 is the prerequisite, whereas increase of 11-KT is the consequence of SSR in tilapia. Furthermore, the continuous increase of serum 11-KT levels at the later stage of SSR indicated that androgens were required for spermatogenesis.

In addition, up-regulation was also observed for the critical factors involved in male pathway, such as *dmrt1*, by transcriptome analysis, qPCR, and IHC during SSR, indicating that the suppression of these genes in the gonad was relieved by the decrease of E2 during SSR. The previous study indicated that *dmrt1* exhibited a male-specific expression in XY gonads from 6 dah, much earlier than the time for morphological differentiation of gonad in tilapia (30), and Dmrt1 suppressed the female pathway by repressing *cyp19a1a* transcription and estrogen production in the testis (32, 56). In protandrous black porgy, the expression of *dmrt1* was limited to the spermatogonia-surrounding cells (Sertoli cells) throughout testis development in the male-fated fish (64). These results indicated that dmrt1 was important not only for testis differentiation in normal development but also for testicular fate determination in NSR. In the present study, Dmrt1-positive signals were detected in the somatic cells surrounding the germ cells at 30-dat TG, when the gonad was basically an ovary, whereas transdifferentiation of germ cell was barely detectable because of the lack of specific spermatogonia marker. This implied that the change of somatic cells probably earlier than that of germ cells. Similarly, the quick up-regulation of *dmrt1* was observed before the appearance of the testicular tissue during PSR in rainbow trout (23). Taken together, these results suggested that up-regulation of *dmrt1* was always associated with the development of testicular tissue in PSR, NSR, and SSR. Dmrt1 played a critical role and could serve as a useful marker for the early development of testicular tissue during PSR, NSR, and SSR.

In conclusion, our study suggests that a differentiated ovary can be transdifferentiated into a functional testis. Down-regulation of endogenous estrogen, which resulted in suppression of female pathway genes and activation of male pathway genes, is the prerequisite for the ovarian transdifferentiation. Up-regulation of endogenous androgen is the consequence of SSR in tilapia.

Acknowledgments

Address all correspondence and requests for reprints to: De-Shou Wang, Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, 400715 Chongqing, People's Republic of China. E-mail address: wdeshou@swu.edu.cn.

This work was supported by National Natural Science Foundation of China Grants 31030063, 91331119, and 31101889, the National Basic Research Program of China Grant 2012CB723205, the National High Technology Research and Development Program (863 Program) of China Grant 2011AA100404, the Program for Changjiang Scholars and Innovative Research Team in University Grant IRT0859, the Specialized Research Fund for the Doctoral Program of Higher Education of China Grant 20090182110008, and the Fundamental Research Funds for the Central Universities Grant XDJK2010C086.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Capel B.** Sex in the 90s: SRY and the switch to the male pathway. *Annu Rev Physiol*. 1998;60:497–523.
2. **Crews D, Bergeron JM, Bull JJ, et al.** Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. *Dev Genet*. 1994;15:297–312.
3. **Nakamura M.** Sex determination in amphibians. *Semin Cell Dev Biol*. 2009;20:271–282.
4. **Devlin RH, Nagahama Y.** Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*. 2002;208:191–364.
5. **Coveney D, Shaw G, Renfree MB.** Estrogen-induced gonadal sex reversal in the tammar wallaby. *Biol Reprod*. 2001;65:613–621.
6. **Scheib D.** Effects and role of estrogens in avian gonadal differentiation. *Differentiation*. 1983;23:S87–S92.
7. **Merchant-Larios H, Ruiz-Ramirez S, Moreno-Mendoza N, Marmolejo-Valencia A.** Correlation among thermosensitive period, estradiol response, and gonad differentiation in the sea turtle *Lepidochelys olivacea*. *Gen Comp Endocrinol*. 1997;107:373–385.
8. **Kobayashi H, Iwamatsu T.** Sex reversal in the medaka *Oryzias lati-*

pes by brief exposure of early embryos to estradiol-17β. *Zoolog Sci*. 2005;22:1163–1167.
9. **Kobayashi T, Kajiura-Kobayashi H, Nagahama Y.** Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. *Cytogenet Genome Res*. 2003;101:289–294.
10. **Piferrer F.** Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture*. 2001;197:229–281.
11. **Guiguen Y, Jalabert B, Thouard E, Fostier A.** Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous seabass, *Lates calcarifer*. *Gen Comp Endocrinol*. 1993;92:327–338.
12. **Chang CF, Lee MF, Chen GR.** Estradiol-17-β associated with the sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. *J Exp Zool*. 1994;268:53–58.
13. **Yeung WS, Chan ST.** The plasma sex steroid profiles in the freshwater, sex-reversing teleost fish, *Monopterus albus* (*Zuiew*). *Gen Comp Endocrinol*. 1987;65:233–242.
14. **Nakamura M, Hourigan TF, Yamauchi K, Nagahama Y, Grau EG.** Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. *Environ Biol Fish*. 1989;24:117–136.
15. **Cardwell JR, Liley NR.** Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. *Gen Comp Endocrinol*. 1991;81:7–20.
16. **Kroon FJ, Liley NR.** The role of steroid hormones in protogynous sex change in the blackeye goby, *Coryphopterus nicholsii* (*Teleostei: Gobiidae*). *Gen Comp Endocrinol*. 2000;118:273–283.
17. **Couse JF, Hewitt SC, Bunch DO, et al.** Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. *Science*. 1999; 286:2328–2331.
18. **Uhlenhaut NH, Jakob S, Anlag K, et al.** Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell*. 2009;139:1130–1142.
19. **Matson CK, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D.** DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature*. 2011;476:101–104.
20. **Paul-Prasanth B, Bhandari RK, Kobayashi T, et al.** Estrogen oversees the maintenance of the femalegenetic program in terminally differentiated gonochorists. *Sci Rep*. 2013;3:2862.
21. **Baron D, Montfort J, Houlgatte R, Fostier A, Guiguen Y.** Androgen-induced masculinization in rainbow trout results in a marked dysregulation of early gonadal gene expression profiles. *BMC Genomics*. 2007;8:357.
22. **Baron D, Houlgatte R, Fostier A, Guiguen Y.** Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens. *Gen Comp Endocrinol*. 2008;156:369–378.
23. **Vizziano D, Baron D, Randuineau G, Mahè S, Cauty C, Guiguen Y.** Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation. *Biol Reprod*. 2008;78:939–946.
24. **Tao W, Yuan J, Zhou L, et al.** Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) reveals differentially expressed genes. *PLoS One*. 2013;8:e63604.
25. **Nakamura M, Nagahama Y.** Differentiation and development of Leydig cells, and changes of testosterone levels during testicular differentiation in tilapia *Oreochromis niloticus*. *Fish Physiol Biochem*. 1989;7:211–219.
26. **Kobayashi T, Kajiura H, Chang X, Nakamura M, Nagahama Y.** Antibodies against trout 3b-hydroxysteroid dehydrogenase: their preparation and immunocytochemical use for fishsteroidogenic tissue. *Zool Sci*. 1996;13:6.
27. **Trapnell C, Williams BA, Pertea G, et al.** Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol*. 2010; 28:511–515.
28. **Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B.** Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*. 2008;5:621–628.
29. **Benjamini Y, Yekutieli D.** Quantitative trait loci analysis using the false discovery rate. *Genetics*. 2005;171:783–790.
30. **Ijiri S, Kaneko H, Kobayashi T, et al.** Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. *Biol Reprod*. 2008;78:333–341.
31. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(−[Delta][Delta]C(T)) method. *Methods*. 2001;25:402–408.
32. **Li MH, Yang HH, Li MR, et al.** Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. *Endocrinology*. 2013;154:4814–4825.
33. **Chang CF, Lau EL, Lin BY.** Stimulation of spermatogenesis or of sex reversal according to the dose of exogenous estradiol-17β in juvenile males of protandrous black porgy, *Acanthopagrus schlegeli*. *Gen Comp Endocrinol*. 1995;100:355–367.
34. **Yeh SL, Kuo CM, Ting YY, Chang CF.** Androgens stimulate sex change in protogynous grouper, *Epinephelus coioides*: spawning performance in sex-changed males. *Comp Biochem Phys C*. 2003; 135:375–382.
35. **Nozu R, Kojima Y, Nakamura M.** Short term treatment with aromatase inhibitor induces sex change in the protogynous wrasse, *Halichoeres trimaculatus*. *Gen Comp Endocrinol*. 2009;161:360–364.
36. **Tsai YJ, Lee MF, Chen CY, Chang CF.** Development of gonadal tissue and aromatase function in the protogynous orange-spotted grouper *Epinephelus coioides*. *Zool Stud*. 2011;50:693–704.
37. **Afonso LO, Wassermann GJ, Terezinha de Oliveira R.** Sex reversal in Nile tilapia (*Oreochromis niloticus*) using a nonsteroidal aromatase inhibitor. *J Exp Zool*. 2001;290:177–181.
38. **Bhandari RK, Alam MA, Soyano K, Nakamura M.** Induction of female-to-male sex change in the honeycomb grouper (*Epinephelus merra*) by 11-ketotestosterone treatments. *Zool Sci*. 2006;23:65–69.
39. **Candi G, Castriota L, Andaloro F, Finoia MG, Marino G.** Reproductive cycle and sex inversion in razor fish, a protogynous labrid in the southern Mediterranean Sea. *J Fish Biol*. 2004;64:1498–1513.
40. **Mackie MC.** Anatomical changes to the gonad during protogynous sex change in the half-moon grouper *Epinephelus rivulatus* (Valenciennes). *J Fish Biol*. 2006;69:176–186.
41. **McBride RS, Johnson MR.** Sexual development and reproductive seasonality of hogfish (Labridae: *Lachnolaimus maximus*), an hermaphroditic reef fish. *J Fish Biol*. 2007;71:1270–1292.
42. **Nakamura S, Kobayashi K, Nishimura T, Higashijima S, Tanaka M.** Identification of germline stem cells in the ovary of the teleost medaka. *Science*. 2010;328:1561–1563.
43. **Kobayashi T, Kajiura-Kobayashi H, Guan GJ, Nagahama Y.** Sexual dimorphic expression of DMRT1 and Sox9a during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). *Dev Dynam*. 2008;237:297–306.
44. **Sudhakumari CC, Senthilkumaran B, Kobayashi T, Wang DS, Chang XT, Nagahama Y.** Expression of cytochrome P-450aromatases in the sex-reversed Nile tilapia. *Fish Physiol Biochem*. 2003; 28:177–178.
45. **Chang X, Kobayashi T, Senthilkumaran B, Kobayashi-Kajura H, Sudhakumari CC, Nagahama Y.** Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). *Gen Comp Endocrinol*. 2005;141:101–115.
46. **Rashid H, Kitano H, Lee KH, et al.** Fugu (*Takifugu rubripes*) sexual differentiation: CYP19 regulation and aromatase inhibitor induced testicular development. *Sex Dev*. 2007;1:311–322.
47. **Heikkilä M, Peltoketo H, Vainio S.** Wnts and the female reproductive system. *J Exp Zool*. 2001;290:616–623.
48. **Uda M, Ottolenghi C, Crisponi L, et al.** Foxl2 disruption causes

mouse ovarian failure by pervasive blockage of follicle development. *Hum Mol Genet*. 2004;13:1171–1181.
49. **Baron D, Cocquet J, Xia X, Fellous M, Guiguen Y, Veitia RA.** An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation. *J Mol Endocrinol*. 2004;33:705–715.
50. **Baron D, Batista F, Chaffaux S, et al.** Foxl2 gene and the development of the ovary: a story about goat, mouse, fish and woman. *Reprod Nutr Dev*. 2005;45:377–382.
51. **Hudson QJ, Smith CA, Sinclair AH.** Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. *Dev Dyn*. 2005;233:1052–1055.
52. **Nakamoto M, Matsuda M, Wang DS, Nagahama Y, Shibata N.** Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun*. 2006; 344:353–361.
53. **Wang DS, Kobayashi T, Zhou LY, et al.** Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. *Mol Endocrinol*. 2007;21:712–725.
54. **Raymond CS, Shamu CE, Shen MM, et al.** Evidence for evolutionary conservation of sex-determining genes. *Nature*. 1998;391:691–695.
55. **Liu Z, Wu F, Jiao B, et al.** Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. *J Endocrinol*. 2007;194:223–241.
56. **Wang DS, Zhou LY, Kobayashi T, et al.** Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. *Endocrinology*. 2010;151:1331–1340.
57. **Yuan J, Tao W, Cheng Y, Huang B, Wang D.** Genome-wide identification, phylogeny, and gonadal expression of fox genes in Nile tilapia, Oreochromis niloticus [published online February 14, 2014]. Fish Physiol Biochem, DOI 10.1007/s10695-014-9919-6.
58. **Zhou LY, Wang DS, Kobayashi T, et al.** A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. *Endocrinology*. 2007;148:4282–4291.
59. **Guiguen Y, Baroiller JF, Ricordel MJ, et al.** Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). *Mol Reprod Dev*. 1999;54:154–162.
60. **Guiguen Y, Fostier A, Piferrer F, Chang CF.** Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *Gen Comp Endocrinol*. 2010;165:352–366.
61. **Kitano T, Takamune K, Nagahama Y, Abe SI.** Aromatase inhibitor and 17α-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). *Mol Reprod Dev*. 2000;56:1–5.
62. **Bhandari RK, Nakamura M, Kobayashi T, Nagahama Y.** Suppression of steroidogenic enzyme expression during androgen-induced sex reversal in Nile tilapia (*Oreochromis niloticus*). *Gen Comp Endocrinol*. 2006;145:20–24.
63. **Navarro-Martín L, Blázquez M, Piferrer F.** Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. *Gen Comp Endocrinol*. 2009;160:3–11.
64. **Wu GC, Chiu PC, Lin CJ, Lyu YS, Lan DS, Chang CF.** Testicular *dmrt1* is involved in the sexual fate of the ovotestis in the protandrous black porgy. *Biol Reprod*. 2012;86:41.