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

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Abstract

To address the roles of doublesex and mab-3-related transcription factor 1 (*Dmrt1*), forkhead transcription factor gene 2 (*Foxl2*), and aromatase in sex differentiation of Southern catfish, the cDNA sequences of these genes were isolated from the gonads. *Dmrt1a* and *Dmrt1b* were found to be expressed in the gonads, being higher in the testis. A low expression level of *Dmrt1b* was also detected in the intestine and kidney of the male. *Foxl2* was found to be expressed extensively in the brain (B), pituitary (P), gill and gonads (G), with the highest level in the ovary, indicating the possible involvement of *Foxl2* in the B–P–G axis. Cytochrome P450 (*Cyp19b*) was found to be expressed in the brain, spleen, and gonads, while *Cyp19a* was only expressed in the gonads and spleen. All-female Southern catfish fry were treated with fadrozole (F), tamoxifen (TAM), and 17 β -estradiol (E₂) respectively, from 5 to 25 days after hatching (dah).

The expression levels of these genes were measured at 65 dah. In the F-, TAM-, and F+TAM-treated groups, *Dmrt1a* and *Dmrt1b* were up-regulated in the gonad, whereas *Foxl2* and *Cyp19a* were down-regulated, while the expression of *Cyp19b* in the gonad remained unchanged. Furthermore, down-regulation of *Foxl2* and *Cyp19b* was also detected in the brain. In the E₂-treated group, *Dmrt1a* and *Dmrt1b* were down-regulated to an undetectable level in the gonad, whereas *Foxl2* and *Cyp19b* were up-regulated in the brain. Consistent with the observed changes in the expressions of these genes, 56, 70, and 80% sex-reversed male individuals were obtained in the F-, TAM-, and F+TAM-treated groups respectively. These results indicate the significant roles of *Dmrt1*, *Foxl2*, and *Cyp19* in the sex differentiation of Southern catfish.

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Introduction

The doublesex and mab-3 (DM)-related transcription factor 1 (*Dmrt1*) belongs to the DM domain gene family. Among the different phyla of the animal kingdom including vertebrates, it is the only gene found to be conserved in structure and function in male sex determination and differentiation. Deletion of a chromosome segment containing *DMRT1* resulted in sex reversal in humans (Raymond *et al.* 1999a). Furthermore, *DMRT1* was found to be expressed in the genital ridge before sex differentiation, specifically in the testis but not in the ovary (De Grandi *et al.* 2000, Shan *et al.* 2000b). Mouse *Dmrt1* was also found to be expressed in the genital ridge during the sensitive period of sex determination and its expression was found to persist during the whole process of

gonad differentiation. However, it was also found to be expressed in both XY and XX embryos, being higher in XY than in XX. Mutation of *Dmrt1* led to failure in producing differentiated testes resulting in germ cell death indicating that *Dmrt1* is a prerequisite for testis differentiation in postnatal mouse (Raymond *et al.* 2000). Also, a number of reports have demonstrated the essential roles of *Dmrt1* in sex determination and differentiation in birds and reptiles (Nanda *et al.* 1999, 2000, Shan *et al.* 2000a, Torres *et al.* 2002, Smith *et al.* 2003). In fish, *Dmrt1* was found to be expressed exclusively during the early stages of male gonad differentiation, but not in the female gonad in Nile tilapia (Guan *et al.* 2000) and rainbow trout (Marchand *et al.* 2000). Further research revealed that treatment with exogenous estrogen down-regulated *Dmrt1* expression in rainbow trout (Marchand *et al.*

2000). However, there is little information on the influence of aromatase inhibitor on *Dmrt1* expression in fish.

Winged helix/forkhead transcription factor gene 2 (*Foxl2*), a member of the forkhead (*FH*) gene family, is a newly isolated transcription factor expressed specifically in the eyelid and adult ovary (Cocquet *et al.* 2003, Loffler *et al.* 2003, Baron *et al.* 2004, Govoroun *et al.* 2004). Mutation of *Foxl2* gives rise to various diseases, such as blepharophimosis-ptosis-epicanthus inversus syndrome and premature ovarian failure in humans (Cocquet *et al.* 2003) as well as polled intersex syndrome (PIS) in goats (Pailhoux *et al.* 2001, 2002, Nikic & Vaiman 2004). During early human development, *Foxl2* plays a crucial role in female reproduction, especially in differentiation and proliferation of granulosa cells, ovarian development, and maintenance of ovarian function by regulating the transcription of certain target genes (Cocquet *et al.* 2003, Loffler *et al.* 2003, Pisarska *et al.* 2004, Yao 2005). Because of its expression in the early genital ridge and its inhibitory action on the male differentiation pathway (Ottolenghi *et al.* 2005), *Foxl2* is recognized as the earliest sex dimorphic marker of ovarian determination and differentiation in mammals. In mice, it is first detected in the developing ovaries at 13.5 days post conception (dpc) and its expression is detected throughout fetal development to the first day of postpartum (Loffler *et al.* 2003). In medaka (*Oryzias latipes*), *Foxl2* is not involved in ovarian determination, but in differentiation of the granulosa cells (Nakamoto *et al.* 2006). In tilapia (*Oreochromis niloticus*), expression of *Foxl2* begins early during differentiation of the gonads and persists until adulthood (Wang *et al.* 2004).

The roles of estrogen in fish reproduction have been particularly well studied because of the diverse reproductive strategies found in this group of vertebrates. The complex effects of estrogen on fish sex differentiation are mediated largely through changes in aromatase activity and expression. As a key enzyme responsible for estrogen synthesis, much research has been focused on this enzyme. Until now, two aromatase genes (*Cyp19a* and *Cyp19b*) have been identified in fish (Chiang *et al.* 2001, Kwon *et al.* 2001). Several reports have demonstrated the involvement of aromatase in fish gonad differentiation (Chang *et al.* 1997, Kitano *et al.* 1999) and oocyte maturation (Bobe *et al.* 2006).

In tilapia, *Cyp19a* was found to be highly expressed in females during early sex differentiation, but dramatically decreased in males between 15 and 27 days after hatching (dah; Kwon *et al.* 2001). A similar situation was found in rainbow trout where aromatase expression was found to be elevated by 100-fold in ovaries before sex differentiation (Guiguen *et al.* 1999). Previous findings strongly indicate that *Cyp19a* plays a decisive role in sex differentiation in these species. However, other reports also demonstrated the important role of *Cyp19b* in zebrafish gonad sex differentiation (Kishida & Callard 2001, Trant *et al.* 2001). It was found that estradiol, ethinylestradiol, and 17-methyltestosterone could enhance the production of *Cyp19b* mRNA in zebrafish embryos. Recent reports on sea bass (Blazquez & Piferrer 2004) also revealed sex-related differences in the expression

profiles of *Cyp19b* in the brain during sex differentiation. These results suggest that *Cyp19b* is also involved in fish sex differentiation. Since *Cyp19a* expression is mainly restricted to the gonads, this gene is probably directly involved in the sex differentiation of the gonads (Chang *et al.* 1997, Guiguen *et al.* 1999, Kitano *et al.* 1999, Kwon *et al.* 2001). On the other hand, since *Cyp19b* is mainly expressed in the brain, this gene is conceivably involved in sex differentiation in an indirect manner (Kwon *et al.* 2001, Chang *et al.* 2005, Kazeto & Trant 2005, Sawyer *et al.* 2006), probably through the brain-pituitary-gonad axis.

Both *Dmrt1* and *Foxl2* are important somatic markers related to the sexual phenotypes and are highly correlated with aromatase activity. Several reports have shown that *Foxl2* and aromatase are co-localized in the somatic cells of the developing XX gonads at both the mRNA and protein levels (Nakamoto *et al.* 2006, Pannetier *et al.* 2006). Further investigations in mammals have indicated that *Foxl2* activates *Cyp19* gene transcription by direct binding to the promoter (Pannetier *et al.* 2006). Our studies on tilapia have also demonstrated that the regulation of *Foxl2* on *Cyp19a* gene transcription is in a female-specific manner (Wang *et al.* 2007). Although located upstream of *Cyp19* and considered the earliest known gene exhibiting sexual dimorphic expression patterns in ovarian somatic cells (Baron *et al.* 2005, Nakamoto *et al.* 2006), information on how *Foxl2* regulates sex differentiation, especially its regulation by other genes essential in sex differentiation, is scarce. Actually some reports in chicken (Hudson *et al.* 2005) have indicated the regulatory action of aromatase on *Foxl2*. Inspired by this study, we have therefore studied the effects of estrogen and aromatase on the expression of *Foxl2* and *Dmrt1* in Southern catfish.

Southern catfish is a good model for studying gonadal sex differentiation in fish. Under laboratory conditions, the fry develop into all-female adult fish without any treatment. Our previous work has revealed the histology of the gonad during the early stages of sex differentiation in this all-female population (Zhang *et al.* 2005). However, the mechanism of this process is still unknown. Here, we report the isolation of *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* cDNAs and their gene expression patterns in Southern catfish. We have also studied the influence of the aromatase inhibitor fadrozole (F), the estrogen receptor antagonist tamoxifen (TAM), and the natural estrogen 17 β -estradiol (E₂) on the expression levels of these genes in this species. The involvement of these genes in sex differentiation in Southern catfish is discussed.

Materials and Methods

Animals

Adult Southern catfish, *Silurus meridionalis* Chen, obtained from the Jia Ling River in Chongqing, a tributary of the Yangtze River, were reared in aerated tanks until use. The fry used in the experiments were obtained by artificial

propagation using parental fish raised in our laboratory. To reduce variations, fry hatched from the same parental fish were used in the experiments. All animal experiments conformed to the Guideline for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

Chemicals and reagents

The steroid hormone (E_2) was purchased from Sigma. The non-steroid aromatase inhibitor (F) and the estrogen receptor antagonist (TAM) were from Novartis Company (AG, Switzerland) and Egis (Budapest, Hungary) respectively. All the primers used in the present study were synthesized by Invitrogen Life Technologies.

Cloning of *Dmrt1a* and *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* cDNAs

Total RNAs from the Southern catfish tissues were prepared using Tripure Reagent (Boehringer, Mannheim, Germany). The first strand cDNA was synthesized with 5 µg total RNA from gonadal tissues using oligo-dT₁₈ primer and M-Mulv reverse transcriptase (Promega) according to the manufacturer's instructions. Using the cDNA as template, degenerate or gene-specific primers were added to the PCR mixtures. The PCR conditions consisted of 94 °C (2 min), followed by 36 cycles of 94 °C (30 s), 57–65 °C (45 s), and 72 °C (1–1.5 min). The reaction was ended by a further 10 min at 72 °C. PCR was performed on a PTC-100 thermal cycler (Bio-Rad). All the primers used in the present study are listed in Table 1. These primers were designed based on the conserved regions of the known *Dmrt1*, *Foxl2*, and *Cyp19* sequences. The PCR products were resolved on a 1.2% agarose gel and the target DNA fragments were purified using QIAquick Gel Extraction Kit (Qiagen). The fragments were then cloned into pGEM-T vector (Promega) and bi-directional sequencing was performed by the dideoxy chain termination method using an ABI PRISM 377 DNA genetic analyzer (Sangon, Shanghai, China).

Then, 5'- and 3'-RACE were performed using the SMART RACE Kit (Clontech) according to the manufacturer's instructions and using the primers (Table 1) designed according to the cloned cDNA fragment sequences. After sequencing of the RACE products, five pairs of primers designed in the untranslated regions (UTR; Table 1) were used to amplify the entire coding regions from the gonad first-strand cDNA. The products were then sequenced again to confirm their DNA sequences.

Sequence analyses

Alignment of nucleotide sequences and their deduced amino acid (aa) sequences was performed using the multiple alignment software DNASTar and ClustalX. ClustalX was also employed to calculate and display the phylogenetic trees using the N-J

method. The values represent bootstrap scores of 1000 trials, indicating the credibility of each branch. All the nucleotide and aa sequences used in the phylogenetic analysis, except those from the Southern catfish, were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>). The GenBank accession numbers of these sequences are as follows: 1) *Dmrt1*: human (AF130728), mouse (NM_015826), dog (XP_851495), chicken (AAF19666), turtle (AB179697), tetraodon (AY152820), fugu (NM_001037949), pejerrey fish (AY319416), platyfish (AF529187), medaka (AF319994), rainbow trout (AF209095), African catfish (AF439561), Nile tilapia (AF203489), black seabream (AY323953), sturgeon (AY057061), zebrafish (NP_991191); 2) *Foxl2*: human (AF301906), goat (AY112725), mouse (AF522275), pig (AY340971), rabbit (AY340972), cattle (AY340970), Tammar wallaby (AY340969), chicken (AY487165), fugu (CAAB01001061), tetraodon (CAG06418), Nile tilapia (AY554172), zebrafish (XM_693823), rainbow trout (AY507927), medaka (AB252055), ciona (BAE06446); 3) *Cyp19a*: sea bass (CAC43178), gilthead seabream (AAL27699), black seabream (AAP23236), red seabream (BAB82524), Atlantic halibut (CAC36394), bastard halibut (BAA74777), Mozambique tilapia (AAD31031), Nile tilapia (AAO62625), channel catfish (AAB32613), goldfish (AAC14013), zebrafish (NP_571229); 4) *Cyp19b*: Nile tilapia (AAO62626), channel catfish (AAL14612), goldfish (AAB39408), zebrafish (NP_571717), fathead minnow (CAC38767); and 5) *Cyp19*: Atlantic stingray (AAF04617), African clawed frog (BAA90529), chicken (AAA48739), American alligator (AAK31803), red-eared slider turtle (AAG09376), mouse (NP_031836), rat (NP_058781), human (NP_112503), monkey (AAK58465), pig (AAB51387), cattle (NP_776730), goat (AAN23836), sheep (CAB40543).

Analysis of *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* expression patterns by RT-PCR

Total RNA (5 µg) was isolated from various tissues of adult Southern catfish, and treated with DNase I (Promega) to remove any possible genomic contamination. All treated total RNA was used to synthesize the cDNA. Reverse transcription was carried out using M-Mulv reverse transcription polymerase (Promega) and total RNAs from adult Southern catfish tissues according to the manufacturer's instructions. The synthesized cDNA was diluted by twofold until use. The quality of the synthesized cDNA was assured by standard RT-PCR together with the negative (in the absence of template), positive (in the presence of the cloned cDNA), and genomic DNA contamination (the template derived from an equal amount of RNA without reverse transcription) controls. Then RT-PCR was employed to analyze the expression patterns of *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b*. The PCR conditions for the target genes consisted of 94 °C (2 min), followed by 28–32 cycles of 94 °C (30 s), 57–65 °C (45 s), and 72 °C (1–1.5 min), ending with 10 min of extension at 72 °C. The following pairs of primers (Table 1)

Table 1 Sequence of primers used in the present study

Primer	Sequence	Purpose
<i>Dmrt1</i> -F1	5'-CTGAAGGGCCACAAGCGCTT-3'	cDNA fragment PCR
<i>Dmrt1</i> -R2	5'-TAGTAGGAGTGCATACGGTACTG-3'	
<i>Foxl2</i> -F2	5'-TG(CT)GAGGA(CT)ATGTT(CT)GAGAAGGG-3'	cDNA fragment PCR
<i>Foxl2</i> -R1	5'-CCCA(GA)TA(TCA)GA(GA)CA(GA)TGCATCAT-3'	
b-F1	5'-TGGATCAA(G)C(T)GGAGAA(G)GAA(G)AC-3'	cDNA fragment PCR
b-R1	5'-TTCAGA(G)ATG(T)TAGGTTG(TC)GTT(GC)CC-3'	
a-F1	5'-TTGAAGCACTCGCAGTACACGT-3'	cDNA fragment PCR
a-R1	5'-TGTATCTCAGTGAGGATGCGT-3'	
<i>Dmrt1</i> -RACE-F1	5'-GGAGACAGCAGGCTCAGGAGGAGGA-3'	RACE
<i>Dmrt1</i> -RACE-F2	5'-GATCGCCTTCTCCAGCAGCACCGGAT-3'	RACE
<i>Dmrt1</i> -RACE-F3	5'-CCTGGTGGTGGATGCTTCTACTACAA-3'	RACE
<i>Dmrt1</i> -RACE-F4	5'-GCTGTCCAGCCACAACATGTCCCAG-3'	RACE
<i>Dmrt1</i> -RACE-R1	5'-TTGTAGTAGGAAGCATCCACCACCAGG-3'	RACE
<i>Dmrt1</i> -RACE-R2	5'-CCGGTGCTGCTGGAGGAAGGCGATCTC-3'	RACE
<i>Dmrt1</i> -RACE-R3	5'-TCCTCCTCTGAGCCTGCTGTCTCCTTA-3'	RACE
<i>Foxl2</i> -RACE-F1	5'-ACCGCCCAACGTACACTTCCAAGC-3'	RACE and tissue distribution
<i>Foxl2</i> -RACE-F2	5'-CCTCTGCGCCCATGTCTACACGTC-3'	RACE
<i>Foxl2</i> -RACE-F3	5'-ACCGGGCTTCAGTTCGCGTGCTCGC-3'	RACE
<i>Foxl2</i> -RACE-R1	5'-GCGAGCACGCGAAGTGAAGCCCGGT-3'	RACE and tissue distribution
<i>Foxl2</i> -RACE-R2	5'-GTGTAGGACATGGGCGCAGGAGGTT-3'	RACE
<i>Foxl2</i> -RACE-R3	5'-GCGAGCACGCGAAGTGAAGCCCGGT-3'	RACE
b-F2	5'-GAGATGGGGAGGCTCGTGGAGCA-3'	RACE and tissue distribution
b-F3	5'-AGGCAGTGTGTGTTGGAGATGGTGA-3'	RACE
b-R3	5'-CACTACACAGCGTAGGAAGTTCAGAGCA-3'	RACE
b-R4	5'-CCTTGTTTCATCCATACCGATGCAC-3'	RACE
b-R5	5'-CTACAGTGAAGCCTTCAATGAC-3'	RACE and tissue distribution
a-F1	5'-TTGAAGCACTCGCAGTACACGT-3'	RACE and tissue distribution
a-F2	5'-CTGCAGAGGACATTGGAATCTGCACC-3'	RACE
a-F3	5'-AACTTGACAACCTGAATTCAGTGAG-3'	RACE
a-R2	5'-TGAAGCTCCTTAGCAGCGTTCTCTG-3'	RACE and tissue distribution
a-R3	5'-TTAGAGCTTTAGTGAAGTACACGCCGCACT-3'	RACE
a-R4	5'-CCCAGTTTACTGCCGAACCTCGACGT-3'	RACE
<i>Dmrt1</i> -UTR-F	5'-AGCAGCAGCAGTAGCACGA-3'	Full-length cDNA amplification and tissue distribution
<i>Dmrt1</i> -UTR-R	5'-GTCAGTCACAGTAACACAGTGCAG -3'	
<i>Dmrt1</i> -ASSR	5'-CTTGACACAGGAGGTCTGTAGTAC -3'	Full-length cDNA amplification
<i>Foxl2</i> -UTR-F	5'-CTTCAGAGGCACTGCGTTTGTTG-3'	
<i>Foxl2</i> -UTR-R	5'-GGTGAGATTCCCGTGTGTTGTT-3'	Full-length cDNA amplification
b-UTR-F	5'-AGCCTGCGAAGTTCAGCCAGACTCACA-3'	
b-UTR-R	5'-GCTTTCATTCTTTATTAGTTTCT-3'	Full-length cDNA amplification
a-UTR-F	5'-ACACAGAAGTTCGGTTCTCCGTTCC-3'	
a-UTR-R	5'-CGCCTTTATCTTCATCCCTCACA-3'	Internal control
β-Actin-F	5'-CCATCTCCTGCTCGAAGTC-3'	
β-Actin-R	5'-CACTGCCCATCTACGAG-3'	

were used to amplify the respective cDNA fragments: *Dmrt1a* (*Dmrt1*-UTR-F and *Dmrt1*-UTR-R giving a 1143 bp cDNA fragment), *Dmrt1b* (*Dmrt1*-UTR-F and *Dmrt1*-ASSR giving a 1221 bp cDNA fragment), *Foxl2* (*Foxl2*-F2 and *Foxl2*-R1 giving a 379 bp cDNA fragment), *Cyp19a* (a-F1 and a-R1 giving a 689 bp cDNA fragment), and *Cyp19b* (b-F1 and b-R1 giving a 404 bp cDNA fragment). A 900 bp Southern catfish β-actin fragment was amplified (28 cycles) using a pair of β-actin primers (Table 1) as the internal control. All the PCR products were resolved on 1·0% agarose gels and then stained with ethidium bromide to visualize the bands.

As members of the DM domain gene family in one species are rather conserved, especially in the DM-domain, the two *Dmrt1* isoforms of Southern catfish are quite similar except at the UTRs. In order to ensure the specificity of the amplification of the two *Dmrt1* isoforms, the forward and reverse primers were designed at the 5'- and 3'-UTRs of the two isoforms respectively. This resulted in relatively large size amplicons. It is known that different size amplicons are amplified at different rates over a PCR run. Therefore, an initial validation study was performed in each case to ensure that the cycle number chosen for each target was within the linear portion of the PCR amplification. In fact the PCR

conditions that we have adopted are very close to similar studies reported in the literature. Generally speaking, the expression levels of transcription factors, such as *Dmrt1* and *Foxl2* are relatively low in the gonad. In the work done on lizard (Sreenivasulu *et al.* 2002) and mouse (Loffler *et al.* 2003), 36 and 35 cycles were used to measure the *Dmrt1* expression level respectively, while in the mouse (Loffler *et al.* 2003) and medaka (Nakamoto *et al.* 2006), 33 cycles and 30–35 cycles were used to measure the *Foxl2* expression levels respectively. As for aromatases, 30–35 cycles were used to measure the expression levels of these genes (Choi *et al.* 2005).

Drug treatment

Southern catfish all-female fry (300 fry/aquarium) were reared in aerated 30×30×50 cm aquaria. The water temperature was kept at 28±1 °C. The drugs (E, TAM and E₂) were dissolved in 95% ethanol and added to the fish feed at the following concentrations: E, 100 µg/g; TAM, 25 µg/g; F+TAM, 100 µg/g+25 µg/g; E₂, 25 µg/g. The vehicle ethanol was added to the control feed. Drug treatment was applied to the fry from 5 to 25 dah, the critical period of sex differentiation in Southern catfish.

Analysis of drug treatment on *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* gene expression levels by semi-quantitative RT-PCR

Brains and gonads were dissected from individual fish of each experimental group at 65 dah. First strand cDNA was prepared from the tissues. The quality of the synthesized cDNA was checked as described in the tissue distribution studies. Semi-quantitative RT-PCR (Zhang & Gui 2004) was performed to measure the mRNA levels using the primers described in the previous section. A series of PCRs with different cycle numbers (from 22 to 36, with an interval of 2) were performed to determine the linear phase of the amplification. Based on these pilot experiments, 28 cycles for β-actin and 30, 32, 28, 30, 32 cycles for the target genes (*Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* respectively) were chosen and applied to the subsequent semi-quantitative RT-PCR analyses. These initial validation studies were essential to ensure the semi-quantitative nature of the mRNA quantitation so that the amount of the amplified product was proportional to the amount of the target template in the samples. Band intensities resulting from the PCR amplification were analyzed using the image analysis software Quantity One (Bio-Rad). *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* mRNA levels were expressed relative to that of β-actin in each sample. In our study, β-actin expression was found to be unaffected by the drug treatment, as reported previously for E₂ (Matsumura *et al.* 2004) and TAM (Parte *et al.* 2002).

Data analyses were performed using one-way ANOVA and the least significant difference on the GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

Histological studies

Gonads of 120 dah fish from the drug treatment groups and the control group were isolated for histological observation as described previously (Zhang *et al.* 2005). Briefly, the gonads were dissected out and fixed in Bouin's solution for 24 h at room temperature, and subsequently dehydrated, embedded in paraffin, and then serially sectioned at 6 µm thickness. The sections were counterstained with hematoxylin–eosin.

Results

Sequences of Southern catfish *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* cDNAs

The isolated *Dmrt1a* cDNA is 1347 bp long, with an open reading frame (ORF) of 891 bp encoding a putative protein of 296 aa (GenBank accession no. EF015487). Southern catfish *Dmrt1a* contains the characteristic 62 aa DNA-binding domain (the DM-domain). It exhibits the highest homology to those isolated from African catfish (78.3%), sturgeon (59.7%) and pejerrey fish (58.6%). It also shares around 48% homology to those isolated from mammals.

The isolated *Dmrt1b* cDNA is 1513 bp long, with an ORF of 816 bp encoding a putative protein of 271 aa (GenBank accession no. EF015488). *Dmrt1b* is a 3'-alternatively spliced form of the *Dmrt1* gene. It contains the same 62 aa DM-domain as in *Dmrt1a*, but with a different C-terminus.

The isolated Southern catfish *Foxl2* cDNA is 1455 bp long, with an ORF of 900 bp encoding a putative protein of 299 aa (GenBank accession no. EF015396) containing the characteristic 105 aa DNA-binding domain (the FH-domain). It shares the highest homology with those isolated from the Nile tilapia (79.9%) and fugu (79.8%), and the lowest homology to that from human (58.3%).

The isolated *Cyp19a* cDNA is 2168 bp long, with an ORF encoding a putative protein of 540 aa (GenBank accession no. AAP83133). The isolated *Cyp19b* cDNA is 2337 bp long, with an ORF encoding a putative protein of 507 aa (GenBank accession no. AAP83132). They share the highest homology with the channel catfish *Cyp19a* (83%) and *Cyp19b* (86%) respectively.

Sequence analyses

Alignment of the Southern catfish *Dmrt1* with those from other vertebrates shows that it is highly conserved, especially in the DM-domain (>86.2% homology), the male-specific motif and the proline/serine (P/S)-rich region near the C-terminus. In addition, the N-termini upstream of the DM-domain and the male-specific motif in Southern catfish

Dmrt1 isoforms are shorter by about 40 aa than the mammalian counterparts (Fig. 1). Based on an alignment of 17 Dmrt1 sequences, a phylogenetic tree was constructed using the fugu Dmrt2 as the outgroup. The high homology of this protein between Southern catfish and African catfish, as well as among other teleosts, is reflected in the tree (Fig. 2).

As shown in Fig. 3, Southern catfish Foxl2 is highly conserved in the FH domain. Outside the FH domain, the C-terminal region is more conserved than the N-terminal region. However, like other non-mammalian counterparts, the Southern catfish Foxl2 contains neither the 14-poly-alanine tract nor the glycine and proline repeats, which are present in all mammalian Foxl2 sequences (Fig. 3). Based on the alignment of 14 complete and 2 partial Foxl2 sequences, a phylogenetic tree was constructed using mouse Foxl1 as the outgroup. The high conservation of this protein in vertebrates, especially among mammals and fish respectively, is reflected in the tree (Fig. 4).

Alignment of the cloned Cyp19 sequences revealed that the Southern catfish aromatases exhibit high homology with the counterparts from other vertebrates. Regions responsible for membrane spanning, α -helix, Ozol's peptides (the steroid substrate-binding domain), aromatic residue-rich, and heme-binding could be identified (Fig. 5). These regions share high homologies with those from other species. It can be seen from the tree (Fig. 6) that the Southern catfish Cyp19a and Cyp19b are clustered into two different clades.

Expression of Dmrt1a, Dmrt1b, Foxl2, Cyp19a, and Cyp19b in Southern catfish tissues

Dmrt1a was found to be exclusively expressed in the gonads, being higher in the testis than in the ovary. Dmrt1b was also expressed predominantly in the gonads, again being higher in the testis than in the ovary. However, a low expression of Dmrt1b was also detected in the intestine and kidney (Fig. 7). On the other hand, Foxl2 was expressed in the brain, pituitary, gill, and gonads, with the highest level in the ovary.

Cyp19a was found to be expressed in the gonads and spleen, being highest in the ovary and lowest in the spleen. No expression was detected in other tissues. Cyp19b was expressed in the brain, gonads, and spleen in both male and female fish, being the highest in the brain and lowest in the spleen (Fig. 7).

Effect of drug treatment on gene expression

The semi-quantitative RT-PCR results demonstrated that Dmrt1a and Dmrt1b mRNA levels were significantly increased

in the gonad (Fig. 8), whereas Foxl2 mRNA levels were decreased by treatment with F, TAM, or F + TAM both in the brain and in the ovary (Fig. 9). On the other hand, Dmrt1a and Dmrt1b mRNA levels in the E₂ treatment group did not exhibit any change (Fig. 8) in comparison with the control group, while Foxl2 mRNA level was increased (Fig. 9).

The expression of Cyp19a mRNA in the gonad, as well as that of Cyp19b in the brain, was significantly down-regulated in groups treated with F, TAM, or F + TAM (Figs 10 and 11). In contrast, the expression of Cyp19b in the gonad of these groups did not show any significant change as compared with the control fish (Fig. 11). On the other hand, the expression of both Cyp19a and Cyp19b mRNA in the gonad was significantly up-regulated when treated with E₂ (Figs 10 and 11).

Gonad histology of the drug-treated fish

At 120 dah, the gonads of the drug-treated fish were studied histologically. Partial sex reversal was observed in the F, TAM, and F + TAM treatment groups. The ovarian walls of these fish were thickened and the ovarian cavity shrunk (Fig. 12D–F). In the F treatment group (Fig. 12D), many degenerating follicles were found and the oocyte number was decreased because of this degeneration, while the numbers of somatic cells were increased as compared with the control (Fig. 12A and B). In the TAM treatment group (Fig. 12E), some cava, which might be the space left behind by the degenerated follicles, were found. Moreover, elongated ellipse-shaped oocytes, which are different from the round- or ellipse-shaped oocytes in the control group, were also found. In the F + TAM treatment group (Fig. 12F), spermatocyst-like structure, spermatogonia, and primary spermatocytes were observed. Complete sex reversal was also observed in the F (56 out of 100 examined fish), the TAM (70 out of 100 examined fish), and the F + TAM (80 out of 100) treatment groups. These sex-reversed gonads showed typical testicular structures (Fig. 12C). On the other hand, the gonad histology of the E₂ treatment fish was the same as the control group.

Discussion

Sequence analysis and tissue distribution of Dmrt1s, Foxl2, and Cyp19s in Southern catfish

In the present study, Southern catfish Dmrt1a and Dmrt1b cDNAs were cloned and the encoded polypeptides were shown to contain the characteristic DM-domain shared by all DM proteins. The DM-domain in Southern catfish Dmrt1a and Dmrt1b exhibited very high homology (>86.2%) with

Figure 1 Alignment of the deduced aa sequences of the Southern catfish Dmrt1 with those from other vertebrates. The sources of the sequences are described in the Materials and Methods section. Regions of high homology are underlined and indicated by Roman numerals: I, the DM-domain; II, the male-specific motif; III, the P/S rich region. The computer programs CLUSTALX and GeneDoc (<http://www.psc.edu/biomed/genedoc>) were used to construct this figure.

	#	20	#	40	#	60	#		
Consensus		MSDDEQTRQPFVDGASGLSPGPV				AKKSPRMPKCSR			
S_catfish_a	:	-----	-----	PNP...AL	-----	-----	H.....	: 35	
S_catfish_b	:	-----	-----	PNP...AL	-----	-----	H.....	: 35	
A_catfish	:	-----	-----	N.K...EV.TP	-----	G.Q	-----	: 35	
zebrafish	:	-----	EE...N	-----	RSLSI	-----	PS.....	: 24	
trout	:	-----	-----	LLECA.PPSA	-----	P	-----	: 32	
medaka	:	-----	KEK.G.P	-----	PEGPAPG	-----	Q	: 29	
tilapia	:	-----	Q.K	-----	PDC.PMSPTK	-----	Q	: 33	
sturgeon	:	-----	MHNS.G.GG	-----	LDCA...AS.T	-----	G.P	: 36	
chicken	:	-----	-----	PAAG	-----	-----	L.L.A	: 15	
mouse	:	T.....V	-----	AGGYSKAAGAMAGA.G.SGA.GS	-----	P.G.S.S	L.A	: 76	
human	:	E.S.....V	-----	AGGFGKASGAL.GA...S.A.GS	-----	G.....S	L.A	: 78	
I									
	80	#	100	#	120	#	140	#	
Consensus	CRNHGYVSPLKGHKRFCNWRDCQCQCKLIAERQVRVMAAQVALRRQQAQEEELGICSPVNL						SGSEIMVKNPEGGDNP		
S_catfish_a	:	F.....	-----	G.C	-----	M.S	-----	PD...K.I	: 110
S_catfish_b	:	F.....	-----	G.C	-----	M.S	-----	PD...K.I	: 110
A_catfish	:	F.....	-----	-----	-----	M.N	-----	D.V.D.N	: 110
zebrafish	:	F.....	-----	R	-----	M	-----	DTL...AV.E	: 99
trout	:	-----	-----	-----	-----	M.L	AT	SQ.VV...T	: 107
medaka	:	F.....	R.K.R.A	-----	G	-----	EAS	P.VT...T.A	: 104
tilapia	:	-----	-----	P	-----	-----	S	M...V.A	: 108
sturgeon	:	-----	T	-----	-----	H	P	PD.DLLI.S.GT	: 113
chicken	:	-----	S	-----	M.K.S	-----	V	SH.P...AP.PV.K-SSSSSS	: 92
mouse	:	-----	A	-----	M.K.S	-----	SH	IP...AA.LL.R.NNAS	: 154
human	:	-----	A	-----	M.K.N	-----	SH	IP...AA.LL.R.NN.S	: 156
I									
	160	#	180	#	200	#	220	#	
Consensus	CLFSVSGSSPSSSTSSAASPAASGSRSVLSDSPTAASRGQHTEGTSDLVVDTSYYSNFYQHPSPRYPAYYSNLYNQ								
S_catfish_a	:	YS.A.A	-----	G.C	-----	S.P	-----	A	: 185
S_catfish_b	:	YS.A.A	-----	G.C	-----	S.P	-----	A	: 185
A_catfish	:	YG.A.A	-----	LA	-----	S.P	-----	A	: 177
zebrafish	:	N...S.G-P	PA	AT	TNL	L.AMS	-----	DC	: 173
trout	:	S	-----	SGGR	CGN	AGT	SNA	G.AS	: 184
medaka	:	-----	EG	-----	G	PGVPPNPLS	A.C.AS	S	: 174
tilapia	:	-----	EG	-----	P	P	H.S.V	AS.P	: 179
sturgeon	:	-----	AP.GG	-----	A.A	P	SSV.E	N.Q	: 188
chicken	:	-----	LQDS	SPAH	-----	VAA	AS	PPEG.M	: 167
mouse	:	-----	MAEN	S.AQPPP	-----	A	TP	EG.M	: 227
human	:	-----	M	EC.G	-----	QPPP	A	VP.T	: 229
I									
	240	#	260	#	280	#	300	#	
Consensus	QYQMPGSDSRSGEVGNTLGGSPVKNSLRSLPAPYVPAQTGNQWQMKNMLSSHNMSSQYRMHSYYSAAASYLSQGLGQG								
S_catfish_a	:	-----	-----	-----	-----	Q	-----	P	: 223
S_catfish_b	:	-----	-----	-----	-----	Q	-----	P	: 223
A_catfish	:	-----	-----	-----	-----	Q	-----	P	: 215
zebrafish	:	-----	G	-----	-----	P	-----	T	: 208
trout	:	-----	N.E	-----	-----	P	-----	S	: 223
medaka	:	-----	PS.G	-----	-----	G.S	-----	P	: 211
tilapia	:	-----	H.G	-----	-----	P	-----	S	: 216
sturgeon	:	-----	QNSE	-----	-----	-----	-----	P	: 225
chicken	:	-----	MAVATE	S.S.T.G.FV	-----	A	-----	T	: 243
mouse	:	-----	SMAL	AE.S	-----	-----	-----	TSE	: 300
human	:	-----	SMALAA	.A	-----	P	-----	G	: 302
II									
	320	#	340	#	360	#	380	#	
Consensus	TAACVPPIFTLEDNNSYTS						EPKASSFSPPSSPSSSQD		
S_catfish_a	:	-----	-----	-----	-----	V	-----	TAA	: 255
S_catfish_b	:	-----	-----	-----	-----	V	-----	TAD	: 247
A_catfish	:	-----	-----	-----	-----	SSVCL	-----	RKLQFHIEMMSPGVK	: 263
zebrafish	:	-----	-----	-----	-----	Q	-----	STCPE	: 230
trout	:	-----	LGQGLGQVLGQGLGGLGHLGQGLGT	-----	-----	TCHD	-----	T.Q	: 284
medaka	:	-----	-----	-----	-----	P	-----	Y	: 240
tilapia	:	-----	-----	-----	-----	TS	-----	F	: 252
sturgeon	:	-----	-----	-----	-----	Q	-----	P	: 257
chicken	:	-----	-----	-----	-----	PT	-----	TQ.LAS	: 275
mouse	:	-----	-----	-----	-----	S	-----	SQ	: 329
human	:	-----	-----	-----	-----	S	-----	QF	: 331
III									
	400	#	420	#					
Consensus	SGLVCLSISSLVNSSTKGVLCESNSSSESGAFTVDSIIEGASKE								
S_catfish_a	:	S.T	-----	P.V	-----	GI	-----	T	: 295
S_catfish_b	:	S	-----	LHL	-----	K-AFTC	-----	G	: 271
A_catfish	:	S.A	-----	P.N	-----	V.AEN	-----	A	: 303
zebrafish	:	-----	DS	-----	-----	AEN	-----	S	: 267
trout	:	D	-----	S	-----	EG	-----	T	: 325
medaka	:	T.T.R	-----	-----	-----	VGV	-----	A	: 279
tilapia	:	T	-----	R	-----	GDA	-----	A	: 292
sturgeon	:	-----	A	-----	-----	G.E	-----	Q	: 298
chicken	:	-----	G	-----	-----	S	-----	E	: 311
mouse	:	-----	S	-----	-----	S	-----	P	: 374
human	:	-----	S	-----	-----	S	-----	P	: 373

Figure 2 Phylogenetic tree of Dmrt1 in vertebrates. The tree was rooted using fugu Dmrt2 (CAC42780) as the outgroup. Branch lengths are proportional to the number of aa changes on the branch. The sources of the sequences are described in the Materials and Methods section.

the counterparts from other vertebrates, suggesting that the DM-domain of Dmrt1 is highly conserved during evolution. In the tissue distribution studies, the Southern catfish *Dmrt1a* is expressed exclusively in the gonads, being higher in the testis than in the ovary. This is consistent with the results reported in other vertebrates (Raymond *et al.* 1999a,b, Guan *et al.* 2000, Kettlewell *et al.* 2000, Marchand *et al.* 2000, Nanda *et al.* 2000, Shibata *et al.* 2002, Torres *et al.* 2002, Smith *et al.* 2003). Due to its early expression in the testis and its high expression level during subsequent gonad development and spermatogenesis, *Dmrt1* is therefore believed to be essential for testis differentiation and maintenance of male characters in vertebrates (Raymond *et al.* 1999a, 2000, Guan *et al.* 2000, Kettlewell *et al.* 2000, Marchand *et al.* 2000, Shibata *et al.* 2002, Smith *et al.* 2003). Results of the present study, therefore, provide supporting evidence of the involvement of *Dmrt1* in male sex differentiation in fish.

We have successfully obtained the 3'-alternatively spliced isoform of *Dmrt1a*, and named it as *Dmrt1b*. The tissue

distribution results demonstrated that *Dmrt1b* is also found in the gonads, again being higher in the testis than in the ovary. However, unlike *Dmrt1a*, which is exclusively expressed in the gonads, *Dmrt1b* is extensively expressed in other non-gonadal tissues including the intestine and kidney of the male fish, indicating that this isoform may have other functions. Some previous reports also demonstrated the expression of *Dmrt1* outside the gonad such as in the chicken Mullerian tract (Shan *et al.* 2000b, Smith *et al.* 2003). The Mullerian tract still belongs to the reproductive system, but the intestine and kidney are not part of the reproductive system. To our knowledge, this is the first report indicating that *Dmrt1* may operate in other non-reproductive systems as well.

In mammals, *Foxl2* is highly expressed in the ovary and is commonly regarded as female-specific (Cocquet *et al.* 2002, Loffler *et al.* 2003, Pannetier *et al.* 2003). In birds, expression of *Foxl2* is predominantly observed in the ovary (Govoroun *et al.* 2004). In fish, studies from tilapia and medaka demonstrated that the expression of *Foxl2* starts in the

	#	20	#	40	#	60	#	80	
Consensus		MMATYPNPEDDAMALMAPETGR		TAEKERAKEEPSPGKGGGGAGT		APKPDPSQKPPYSYVALIAMAI		RESAEKRLTL	SG
S_catfish	:	...A.LGL.ES		TA.G.DTA.--K...QI.I.SVAV		-----SA..A.A.....D.S.....			: 70
zebrafish	:	GH...G.I.DTT.S-		...D.T.D.AP.E.-----G.D.S.....					: 71
fugu	:	Q.....VHD.NT.K...		P...VQD.-----VE.....S.....					: 72
tilapia	:	Q.....IHD.NT.K...		P...VQD.-----VS.....S.....					: 72
trout	:	...D..Q.....VHD.NMAKD..P...		VQE.-----VS..T.....T.....					: 72
chicken	:	..SG.A.G.E.....HDG.G.K.P..G...		L.AE.-----G.....					: 72
pig	:	E...A.G.....KEPE.PPPL.....		S.....A.....					: 80
cattle	:	E...A.SG.....A.KEPE.PPP.....		T.....T.....A.....					: 80
mouse	:	E...T.GT.....AVKEAE.SPP-		T.....A.....					: 75
human	:	E...A.G.....VKEPEGPPP-		G.....A.....					: 79
	#	100	#	120	#	140	#	160	
Consensus		IYQYIISKFFFYKKNKGWQNSIRH		NLSNECFIKVREGGGERKGN		YWTLDPA		CEDMFEKGN	YRRRRRMKRFRRPPPTH
S_catfish	:								: 150
zebrafish	:								: 151
fugu	:								: 152
tilapia	:								: 152
trout	:								: 152
chicken	:								: 152
pig	:	A.....							: 160
cattle	:	A.....							: 160
mouse	:	A.....							: 155
human	:	A.....							: 159
	I	#	180	#	200	#	220	#	240
Consensus		FQPGKSLFGAGGAAGGCGVAGAG		GDGYLYLSPPKYLQSGFMNNS		WLPQPPSPMPYASQMASGNV		SPVNAAAAAGPGSP	
S_catfish	:	..A.....		S.....P.....S.A..A.S.T.....					: 206
zebrafish	:			E.....S-----A.S.T...S.S.....					: 203
fugu	:					S...S.G..A.S.T.....			: 208
tilapia	:	..A.....		S.....S.....S.G...S.T.....					: 208
trout	:			S.....S.G...S.T.....					: 208
chicken	:			P.....T.....A.....SG.S.....					: 208
pig	:	G.....A.....A.....						AAAAAAA	: 240
cattle	:	G.....A.....A.....						AAAAAAA	: 240
mouse	:	G..S.....P...A.....						AAAAAAA	: 235
human	:	G.....A.....A.....						AAAAAAA	: 239
	#	260	#	280	#	300	II	320	
Consensus		GAAAVVKGLSAPSSSYGPFYSRVQ		SMALPPGMVNSYNGMSGPPA		APPPPPPPHPPHPPHHHLHHA		APPPAPPHHGAA	
S_catfish	:	-----H...N.....T...A.....S.....					THQ		: 245
zebrafish	:	-----N.....G...S.....					H..THP-----H.L		: 248
fugu	:	-----N.....S.....					PA-----P		: 248
tilapia	:	-----N.....S.....						P	: 245
trout	:	-----N.....G...S.....					P-----		: 247
chicken	:	-----G.A.-.....-.....A.....					P..P.A-----		: 247
pig	:	AG.AA.....		G.....S.....A.....A.....					: 317
cattle	:	AG.AA.....		G.....S.....A.....A.....					: 317
mouse	:	AG.AA.....		G.....A.....A.....					: 315
human	:	AG.AA.....		G.....A.....A.....					: 316
	#	340	#	360	#	380	III		
Consensus		PHPQQLSPASPATAAPPVSTTNG		AGLQFACARQPAELSMHCSY		WDHDSKHGALHSRIDI			
S_catfish	:	--H.....S.P..AV..T.....S...T..P...P..E.EG..S.....							: 299
zebrafish	:	..A.....A.P..G..T.....S.....				E...S...A...			: 306
fugu	:	H.A.....P.....S.....				E.E...S.....			: 306
tilapia	:	H.T.....P.....S.....				E.E...S.....			: 303
trout	:	H.A...N...V...P.....S...T.....				E...S...A...			: 305
chicken	:	H...G...P...APAA.....				E.....			: 305
pig	:	..P.G.....PAP..AP.....A.....T.....							: 377
cattle	:	..P.G.....PAP..AP.....A.....T.....							: 377
mouse	:	..P.G.....PAP.SAP.....A.....T.....							: 375
human	:	..P.G.....PAP.SAP.....A.....T.....							: 376

Figure 3 Alignment of aa sequences of the Southern catfish Foxl2 with those from other vertebrates. The sources of the sequences are described in the Materials and Methods section. Regions of high homology are underlined and indicated by Roman numerals: I, the FH domain; II, the polyalanine tract; III, the glycine and proline repeats. The computer programs CLUSTALX and GeneDoc (<http://www.psc.edu/biomed/genedoc>) were used to construct this figure.

Figure 4 Phylogenetic tree of Foxl2. The tree was rooted using mouse Foxl1 (NM_008024) as the outgroup. Branch lengths are proportional to the number of aa changes on the branch. Partial sequences (*) may yield artificial short branches. The sources of the sequences are described in the Materials and Methods section.

somatic cells surrounding the germ cells in XX gonads immediately after initiation of ovarian differentiation and is maintained in the granulosa cells throughout ovarian development. In adult ovary, *Foxl2* is expressed in the previtellogenic and vitellogenic follicles, but its expression ceases in the postvitellogenic follicles (Wang *et al.* 2004, Nakamoto *et al.* 2006). In the present study, the tissue distribution results revealed that the Southern catfish *Foxl2* expression is restricted to the brain (B), pituitary (P), gill, and gonads (G). This is consistent with our previous findings in

tilapia (Wang *et al.* 2004). This expression pattern indicates that the B–P–G axis and the hormone synthesizing enzymes might be the main targets of *Foxl2*. Moreover, our results also demonstrated a lower expression level of *Foxl2* in the testis. Similar results have been reported in tilapia and rainbow trout (Baron *et al.* 2004, Wang *et al.* 2004). These results indicate that *Foxl2* might also play some roles in the male fish as well. In the present study, the cDNAs of two types of aromatase have been isolated in Southern catfish. Homology analysis showed that they are highly conserved even though they are

Figure 5 Alignment of the deduced aa of the Southern catfish Cyp19s with those other teleosts. The sources of the sequences are described in the Materials and Methods section. Regions of high homology are indicated by Roman numerals: I, the membrane spanning domain; II, the helical region; III, Ozol’s peptide; IV, the aromatic region; V, the heme-binding region. Asterisks and dots indicate identical and similar aa residues respectively. The computer programs CLUSTALX and GeneDoc (<http://www.psc.edu/biomed/genedoc>) were used to construct this figure.

Figure 6 Phylogenetic tree of Cyp19 in vertebrates. The tree was rooted using fruit fly CYPIVD2 as the outgroup. Branch lengths are proportional to the number of aa changes on the branch. The sources of the sequences are described in the Materials and Methods section.

encoded by two different genes. Tissue distribution analysis revealed that both *Cyp19a* and *Cyp19b* were expressed in the gonads and spleen, whereas *Cyp19b* was also expressed in the brain. Gonad and brain are the major tissues of estrogen production in fish as well as in other vertebrates. The high expression of both types of aromatase in the ovary and *Cyp19b* in the brain has been reported in several teleosts (Trant *et al.* 1997, Tchoudakova & Callard 1998, Kitano *et al.* 1999, Kwon *et al.* 2001). In our study, *Cyp19a* was not detected in the brain, similar to the situation found in zebrafish (Kishida & Callard 2001). In line with our findings, *Cyp19a* was also reported to be expressed in the spleen of tilapia (Chang *et al.* 2005). Spleen is not a steroidogenic tissue. Nevertheless, aromatase and *P450_{scc}* (cholesterol side-chain cleavage enzyme) were found to be expressed in the spleen of human and mouse (Price *et al.* 1992, Morohashi *et al.* 1999). Screening of the major steroidogenic enzymes is needed to ascertain whether the teleost spleen is capable of carrying out the steroidogenesis process or not. However, our data indicate that at least the circulating steroid precursors could be converted to estrogen in this organ. Another interesting point worthy of further investigation is the reason for the need to synthesize steroids/estrogen in the spleen.

Regulatory action of estrogen on *Dmrt1s*, *Foxl2*, and *Cyp19s* in Southern catfish

In the present study, the all-female Southern catfish fry were treated with an aromatase inhibitor (F) and an estrogen receptor antagonist (TAM) during the sensitive period of sex differentiation (5–25 dah; Zhang *et al.* 2005). Expression of the Southern catfish *Dmrt1a* and *Dmrt1b* in the gonad was found to be the same in the E₂ treatment group as in the control group, whereas substantial up-regulation was detected in the F, TAM, and F+TAM groups (Fig. 8). While *Foxl2* expression in the gonads and brain was significantly up-regulated in the E₂ treatment group, it was down-regulated in the F, TAM and F+TAM groups (Fig. 9). Simultaneously, *Cyp19a* expression in the gonads and *Cyp19b* expression in the brain were also decreased in the F, TAM, and F+TAM groups (Figs 10 and 11).

Several reports have revealed the regulatory action of estrogen on the expression of *Dmrt1*, *Foxl2*, and aromatase (Marchand *et al.* 2000, Kishida & Callard 2001, Kishida *et al.* 2001, Tsai *et al.* 2001, Baron *et al.* 2004, 2005, Menuet *et al.* 2005). In the work done on rainbow trout, *Dmrt1* (Marchand *et al.* 2000) and *Foxl2* (Baron *et al.* 2004) expression was down-regulated and up-regulated respectively when the fish

Figure 7 RT-PCR analysis of *Dmrt1a*, *Dmrt1b*, *Foxl2*, *Cyp19a*, and *Cyp19b* gene expression in various tissues of the adult Southern catfish. B, brain; P, pituitary; G, gill; H, heart; S, spleen; L, liver; I, intestine; O, ovary; K, kidney; M, muscle; T, testis; +, positive control; –, negative control. β -Actin was used as the internal control.

was treated with estrogen, whereas in zebrafish and tilapia, estrogen treatment could up-regulate aromatase expression (Kishida & Callard 2001, Kishida *et al.* 2001, Tsai *et al.* 2001, Menuet *et al.* 2005). Other reports have shown that reduction in estradiol amount (by F treatment) increased *Dmrt1* expression (Smith *et al.* 2003) as well as decreased *Foxl2* (Hudson *et al.* 2005) and aromatase expression (Kishida & Callard 2001, Kishida *et al.* 2001, Tsai *et al.* 2001, Menuet *et al.* 2005) and enzyme activity (Melo & Ramsdell 2001, Lee *et al.* 2004). In the present study, both reduction in estrogen amount (by F treatment) and inhibition of estrogen signaling (by TAM treatment) resulted in up-regulation of *Dmrt1* expression but down-regulation of *Foxl2* and aromatase expression. On the other hand, E_2 treatment caused down-regulation of *Dmrt1* expression and up-regulation of *Foxl2* expression. These results provided additional evidence for the important role of estrogen on the expression of *Dmrt1*, *Foxl2*, and aromatase. However, there is no putative ER-binding site or estrogen response element (ERE) observed on the promoters of known *Dmrt1* and *Foxl2* genes. Therefore, the down-regulation of *Dmrt1* and up-regulation of *Foxl2* by estrogen treatment might be an indirect effect. This indirect regulatory action of estrogen on *Foxl2* expression might be mediated through a positive feedback mechanism as suggested in chicken (Hudson *et al.* 2005). Our results support this hypothesis, indicating the conservation of this regulatory mechanism among vertebrates. In our results, these regulatory phenomena were also observed in the brain, indicating that sex differentiation was controlled at different levels in the B–P–G axis.

Previous promoter analysis showed that the binding sites for several sex determining factors in mammals such as Ad4BP/SF-1, WT1, and SRY were present in the 5'-flanking region of *Cyp19a* but not *Cyp19b*. On the other hand, ERE was found only in the 5'-flanking region of *Cyp19b* but not *Cyp19a* in fish, indicating that these two aromatase genes might be regulated differently (Callard *et al.* 2001, Kazeto *et al.* 2001, Tchoudakova *et al.* 2001, Tong & Chung 2003, Chang

et al. 2005, Kazeto & Trant 2005). In the present study, E_2 treatment up-regulated *Cyp19b* in the brain and *Cyp19a* in the gonad. However, E_2 treatment only caused a mere 1.4-fold up-regulation of *Cyp19b* in the brain, lower than the results reported by others (Menuet *et al.* 2005, Sawyer *et al.* 2006) where dramatic up-regulation of *Cyp19b* by E_2 treatment was reported both *in vitro* and *in vivo*. Elevated expression of *Cyp19b* was also observed in channel catfish and zebrafish embryos by estrogen treatment (Kishida & Callard 2001, Trant *et al.* 2001, Blazquez & Piferrer 2004). These discrepancies might be explained by the different time points employed for gene expression evaluation after drug treatment.

Figure 8 Regulation of *Dmrt1a* (white column) and *Dmrt1b* (black column) gene expression in Southern catfish gonad by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values $\pm$ S.E.M. from five individual fishes. (** $P < 0.01$ as compared with the respective control by one-way ANOVA). C, control; E_2 , 17 β -estradiol; F, fadrozole; TAM, tamoxifen; F+TAM, fadrozole+tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

Figure 9 Regulation of *Foxl2* gene expression in Southern catfish brain (white column) and gonad (black column) by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values $\pm$ S.E.M. from five individual fishes. (* $P < 0.05$; ** $P < 0.01$ as compared with the respective control by one-way ANOVA). C, control; E₂, 17 β -estradiol; F, fadrozole; TAM, tamoxifen; F + TAM, fadrozole + tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

The dramatic up-regulation of *Cyp19b* during E₂ treatment is an acute event that occurs quickly. The presence of ERE in the *Cyp19b* promoter of channel catfish (Kazeto & Trant 2005) and zebrafish (Kazeto *et al.* 2001, Tchoudakova *et al.*

Figure 10 Regulation of *Cyp19a* gene expression in Southern catfish gonad by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values $\pm$ S.E.M. from five individual fishes. (* $P < 0.05$; ** $P < 0.01$ as compared with the respective control by one-way ANOVA). C, control; E₂, 17 β -estradiol; F, fadrozole; TAM, tamoxifen; F + TAM, fadrozole + tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

Figure 11 Regulation of *Cyp19b* gene expression in Southern catfish brain (white column) and gonad (black column) by various drug treatments. Results obtained by semi-quantitative RT-PCR are expressed as mean values $\pm$ S.E.M. from five individual fishes. (* $P < 0.05$; ** $P < 0.01$ as compared with the respective control by one-way ANOVA). C, control; E₂, 17 β -estradiol; F, fadrozole; TAM, tamoxifen; F + TAM, fadrozole + tamoxifen. The doses of the drugs used are described in the Materials and Methods section.

2001) supports this notion. In our case, the gene expression levels were checked at 65 dah, 40 days after the termination of E₂ treatment (which lasted from 5 to 25 dah). At 65 dah, the peak of the E₂-stimulated *Cyp19b* expression is probably already passed but the subsequent biological effects of this E₂-stimulated gene expression are still prominent.

In contrast to the up-regulation of *Cyp19b* expression in the brain after E₂ treatment, *Cyp19b* expression in the gonad remains unchanged. This raises the following possibility in explaining this differential expression pattern in Southern catfish: either tissue-specific promoters are employed for *Cyp19b* transcription in the gonad and brain as in the case of human or perhaps only in the brain is there an appropriate cellular context for transcriptional activation by estrogen. Further studies on this interesting point are highly warranted.

The possible roles of Dmrt1s, Foxl2, and Cyp19s in the sex differentiation of Southern catfish

Different from mammals, sex determination and differentiation of non-mammalian vertebrates depend less on the sex determination genes. On the contrary, hormones and environmental factors play very important roles. Among these factors, estrogen plays key roles in ovarian differentiation in non-mammalian vertebrates, including fish (Chang *et al.* 1997, Kwon *et al.* 2000, Lee *et al.* 2000, Kobayashi *et al.* 2003), amphibians (Yu *et al.* 1993), and birds (Elbrecht & Smith 1992, Smith & Sinclair 2004). As the key enzyme catalyzing the conversion of endogenous androgen to

Figure 12 Histological observation of control and drug-treated Southern catfish gonads. (A) Ovary of control fish at 120 dah; scale bar = 100 μm . (B) Part of A with high magnification; scale bar = 50 μm . (C) Typical testis observed in the fadrozole (F)-, tamoxifen (TAM)-, and F + TAM-treated fish; scale bar = 50 μm . (D–F) Ovotestis observed in the F-, TAM-, and F + TAM-treated fish respectively; scale bar = 50 μm . The inserts in D, E, F show the waned ovary cavities for the three drug-treated groups respectively. PO, primary oocyte; OC, ovary cavity; ST, Sertoli cells; AO, atretic oocyte; CA, cavum; SC, spermatocyte; SG, spermatogonia; ST-L, spermatocyst-like structure.

estrogen, aromatase is highly related to gonad malformation, sterility, intersex, or sex reversal. Inhibition of aromatase gene expression and enzyme activity resulted in reduced estrogen production and subsequently male-to-female sex reversal, which has been reported in Coho salmon (*Oncorhynchus kisutch*; Afonso *et al.* 1999), bastard halibut (*Paralichthys olivaceus*; Kitano *et al.* 2000), and Nile tilapia (Kobayashi *et al.* 2003). Consequently, the maintenance of aromatase expression and activity is the prerequisite for ovarian differentiation and development.

Until now, several genes have been reported to be involved in aromatase regulation and sex differentiation of vertebrates, including *Dmrt1* and *Foxl2* through interaction with Ad4BP/SF-1.

The role of *Dmrt1* in sex determination and differentiation of vertebrates has been studied in several species (Nanda *et al.* 1999, 2000, Raymond *et al.* 1999a,b, Kettlewell *et al.* 2000, Marchand *et al.* 2000, Shan *et al.* 2000a, Smith *et al.* 2003). In mice, humans, chicken, and turtles, *Dmrt1* expression was found to be sexually dimorphic and restricted to the testis (Raymond *et al.* 1999a,b, Kettlewell *et al.* 2000, Shan *et al.* 2000a, Smith *et al.* 2003). Moreover, *Dmrt1*^{-/-} knockout XY mice develop testicular differentiation failure (Raymond *et al.* 2000), while humans with chromosome 9p deletion develop sex reversal (Raymond *et al.* 1999a). In chicken treated with aromatase inhibitor, *Dmrt1* expression increased significantly. Aromatase inhibitor-treated ZW embryos

endured sex reversal and developed as physiological males (Smith *et al.* 2003). These results suggest the important role of *Dmrt1* in male sex determination and differentiation of vertebrates.

Study of has Nile tilapia further revealed that *Dmrt1* can suppress Ad4BP/SF-1- and Foxl2-mediated *Cyp19a1* transcription. *Dmrt1* can also suppress Ad4BP/SF-1-mediated *StAR* and *p450c17*, but activate *Cyp11a1* and *Cyp11b* promoter activities. These results suggest that *Dmrt1* plays a decisive role in the testicular differentiation of Nile tilapia by down-regulating aromatase gene expression and possibly by shifting the entire steroidogenic pathway towards androgen production (Wang *et al.* 2006).

In the present study, Southern catfish *Dmrt1a* and *Dmrt1b* were found to be expressed mainly in the gonads. In addition, the expression of *Dmrt1a* and *Dmrt1b* was decreased by E₂ treatment but increased by E, TAM, or F + TAM treatment. Consistent with the increased expression of *Dmrt1a* and *Dmrt1b*, 50, 70, and 80% of sex-reversed male fish were obtained in the F, TAM, or F + TAM treatment groups respectively, while the expression of both *Dmrt1a* and *Dmrt1b* remained low in the female control (Fig. 8). This demonstrates that the expression of *Dmrt1a* and *Dmrt1b* is highly related to the male phenotype and reveals the pivotal role *Dmrt1* plays in Southern catfish testicular differentiation, probably mediated through regulating aromatase expression.

To date, no upstream regulators or downstream targets of *Dmrt1* have been identified, making it difficult to place *Dmrt1* in any gene regulatory pathway. Even though our data and those from others suggest a potential regulatory linkage between *Dmrt1* and *Cyp19*, further investigations are necessary to establish their casual relationship.

Foxl2 has been shown to be involved in ovarian differentiation in previous reports (Baron *et al.* 2004, Ottolenghi *et al.* 2005, Nakamoto *et al.* 2006). In mouse, chicken, and turtle, representatives of three phylogenetically distant vertebrate groups that possess different mechanisms of sex determination, the expression of *Foxl2* was detected in the early ovaries of all these three species around the time of sex determination and *Foxl2* expression was sexually dimorphic in all cases (Loffler *et al.* 2003). These data suggested that *Foxl2* is a highly conserved early regulator of vertebrate ovarian differentiation. In fish, *Foxl2* expression was found to be sexually dimorphic in Nile tilapia (Wang *et al.* 2004, 2007), rainbow trout (Baron *et al.* 2004), and medaka (Nakamoto *et al.* 2006), supporting the hypothesis that *Foxl2* may play important role in ovarian differentiation in teleosts as well.

Further evidence for the involvement of *Foxl2* in the transcriptional regulation of *Cyp19* was reported recently in vertebrates. In goats suffering from PIS, *Foxl2* was found to activate the *Cyp19* specific promoter 2, and *Cyp19* expression was reduced when *Foxl2* ceased to express in the primary stages of ovarian differentiation (Pannetier *et al.* 2006). In the early stages of gonad differentiation, *Foxl2* and Ad4BP/SF-1 were found to be co-localized with *Cyp19a* in the ovary of tilapia. Promoter assays further revealed that the FH-domain of *Foxl2* binds to the consensus ACAAATA sequence on the *Cyp19a* promoter, thereby activating the expression of *Cyp19a*. Furthermore, *Foxl2* can interact with Ad4BP/SF-1 to enhance Ad4BP/SF-1-activated *Cyp19a* gene transcription in a female-specific manner (Wang *et al.* 2007).

In our study, the expression of *Foxl2* was found to be sexually dimorphic with dominant expression in the ovary, consistent with previous reports (Loffler *et al.* 2003, Baron *et al.* 2004, Wang *et al.* 2004, Nakamoto *et al.* 2006). Furthermore, *Cyp19a* and *Cyp19b* were also found to be expressed mainly in the ovary and brain respectively, being higher in female than in male. When treated with F and/or TAM, *Foxl2/Cyp19a* expression in the gonad and *Foxl2/Cyp19b* expression in the brain decreased significantly, similar to the results reported in chicken (Hudson *et al.* 2005). Gene expression study and histological analyses of the F-, TAM-, and F+TAM-treated fish revealed that the down-regulation of *Foxl2*, *Cyp19a*, and *Cyp19b* expression was highly correlated with the female-to-male sex reversal of Southern catfish in these groups. These results, together with the tissue distribution of *Foxl2*, *Cyp19a*, and *Cyp19b* mRNA in Southern catfish, strongly suggest that these genes are involved in Southern catfish ovarian differentiation and development. Furthermore, the down-regulation of *Foxl2* and *Cyp19b* in the brain of the F and/or TAM treatment

groups also indicated that *Foxl2* and *Cyp19b* might regulate sex differentiation at the brain and pituitary levels. It is well known that brain sexualization in mammals is, at least in part, determined early in development through the testis-differentiating effects of *Sry*, whereas fish could retain the possibility of constantly adapting their brain gender to their gonadal gender. For these reasons, *Cyp19b* appears to be a critical gene whose disruption could grossly affect the functioning of the whole reproductive axis (Menuet *et al.* 2005).

Consistent with the changes in gene expression patterns in the drug-treated fish, obvious morphological changes, from partial to complete sex reversal, were also observed in the gonads of the F, TAM, and F+TAM treatment groups as compared with control fish. These features include incomplete/diminished ovarian cavity formation, elongation of the oocytes, degeneration and reduction of the follicles, proliferation of somatic cells, and appearance of testicular structures (Zhang & Xie 1996), such as spermatogonia, primary spermatocytes, etc. These results suggest that F and/or TAM treatment shrink the ovarian cavities in size probably by compression of the thickened ovary wall, as reported in golden rabbitfish (*Siganus guttatus*; Komatsu *et al.* 2006). However, F, TAM, and F+TAM treatment resulted in only 56, 70, and 80% fish being sex-reversed from female to male. On the other hand, 100% sex-reversed phenotypic males were reported in fathead minnow (*Pimephales promelas*; Zerulla *et al.* 2002) and zebrafish (Fenske & Segner 2004). Despite that 100% males were observed in the F-treated zebrafish, only 64% of the fish showed male-phenotypical expression of *Cyp19a*, indicating that the physiological regulation of estradiol synthesis was not irreversibly masculinized by the F treatment (Fenske & Segner 2004). In our study, though the F-, TAM-, and F+TAM-treated fish showed very low expression of *Cyp19a*, only 56, 70, and 80% males resulted respectively. The reason why all Southern catfish offspring obtained by artificial propagation under laboratory conditions are female remains elusive, making it unresolved at the moment why we were unable to generate 100% sex-reversed males in the F, TAM, and F+TAM treatment groups. Furthermore, since sex-linked genomic markers are not yet available for Southern catfish, it is not possible at this stage to ascertain whether those fish, which are still undergoing transition from female to male in the drug treatment groups, are genetic females or not.

In summary, the cDNAs of four genes, which showed clear sexual dimorphic expression pattern from fry to adult, were cloned from the Southern catfish. *Foxl2*, *Cyp19a* (gonad only), and *Cyp19b* are expressed higher in the female brain and gonad, thus favoring ovarian differentiation. On the other hand, *Dmrt1a* and *Dmrt1b* are expressed higher in the male gonad, thus favoring male sex differentiation. Treatment with F, TAM, and F+TAM of the all-female fry reversed the expression patterns of these genes and caused sex reversal of the treated fish. Taken together, our results indicate that *Dmrt1*, *Foxl2*,

Cyp19a, and *Cyp19b* are important genes implicated in the sex differentiation of Southern catfish.

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