Molecular cloning of doublesex and mb-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

Zhihao Liu1*, Fengrui Wu1*, Baowei Jiao1,3,*, Xiuyue Zhang1, Chongjiang Hu1, Baofeng Huang1, Linyan Zhou1,2, Xigui Huang1, Zhijian Wang1, Yaoguang Zhang1, Yoshitaka Nagahama2, Christopher H K Cheng3 and Deshou Wang1

1Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing 400715, China
2Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan
3Department of Biochemistry and the Environmental Science Programme, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China

Abstract

To address the roles of doublesex and mb-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 (Cyp)19b 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 17β-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 Cyp 19a were down-regulated, while the expression of Cyp 19b in the gonad remained unchanged. Furthermore, down-regulation of Foxl2 and Cyp 19b 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 Cyp 19b 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 Cyp 19b in the sex differentiation of Southern catfish.


Introduction

The doublesex and mb-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. 2000a). 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-p toes-epicanthus inversus syndrome and premature ovarian failure in humans (Cocquet et al. 2003) as well as polled intersex syndrome (PIS) in goats (Pailloux 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 17β-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 (E2) 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 (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 dyeosexy 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), zebrasfish (NP_991191); 2) Foxl2: human (AF301906), goat (AY127257), mouse (AF522275), pig (AY340971), rabbit (AY340972), cattle (AY340970), Tammar wallaby (AY340969), chicken (AY487165), fugu (CAAB01001061), tetraodon (CAG06418), Nile tilapia (AY554172), zebrasfish (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 (AUC14013), zebrasfish (NP_571229); 4) Cyp19b: Nile tilapia (AAO62626), channel catfish (AAL14612), goldfish (AAB39408), zebrasfish (NP_571717), fathead minnow (CAC38767); and 5) Cyp19: Atlantic stingray (AAF04617), African clawed frog (BA90529), chicken (AAA48739), American alligator (AAK31803), red-eared slider turtle (AAO9376), mouse (NP_031836), rat (NP_058781), human (NP_112503), monkey (AAK58465), pig (AABS387), 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.
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), Cyp19a1a (a-F1 and a-R1 giving a 689 bp cDNA fragment), and Cyp19b1 (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% 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

Table 1  Sequence of primers used in the present study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dmrt1-F1</td>
<td>5'-CTGAGGGCCCAACGCAGCGT-3'</td>
<td>cDNA fragment PCR</td>
</tr>
<tr>
<td>Dmrt1-R2</td>
<td>5'-TACGCTCAAGCTTATGACTGCT-3'</td>
<td>cDNA fragment PCR</td>
</tr>
<tr>
<td>Foxl2-F2</td>
<td>5'-TGCTCAAGCTTATGACTGCT-3'</td>
<td>cDNA fragment PCR</td>
</tr>
<tr>
<td>Foxl2-R1</td>
<td>5'-CTGAGGGCCCAACGCAGCGT-3'</td>
<td>cDNA fragment PCR</td>
</tr>
<tr>
<td>b-F1</td>
<td>5'-CCCAAGACTGCAGTACAC-3'</td>
<td>RACE</td>
</tr>
<tr>
<td>b-R1</td>
<td>5'-GATGAACTGCAGTACAC-3'</td>
<td>RACE</td>
</tr>
<tr>
<td>a-F1</td>
<td>5'-ACCAAGACTGCAGTACAC-3'</td>
<td>RACE</td>
</tr>
<tr>
<td>a-R1</td>
<td>5'-CCCAAGACTGCAGTACAC-3'</td>
<td>RACE</td>
</tr>
<tr>
<td>Dmrt1-RACE-F1</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-RACE-F2</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-RACE-F3</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-RACE-F4</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-RACE-R1</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-RACE-R2</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-RACE-R3</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-UTR-F</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Dmrt1-UTR-R</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Foxl2-UTR-F</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>Foxl2-UTR-R</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>a-UTR-F</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>a-UTR-R</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>β-Actin-F</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
<tr>
<td>β-Actin-R</td>
<td>5'-AGCAGCGAACGCTGAGTACAC-3'</td>
<td>Full-length cDNA amplification and tissue distribution</td>
</tr>
</tbody>
</table>

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), Cyp19a1a (a-F1 and a-R1 giving a 689 bp cDNA fragment), and Cyp19b1 (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% 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 (F, TAM and E2) were dissolved in 95% ethanol and added to the fish feed at the following concentrations: F, 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 those isolated from mammals.

The isolated Cyp19a cDNA is 2168 bp long, with an ORF of 1567 bp encoding a putative protein of 519 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-polyalanine 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 Fox1 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 cavity, 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...
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,b, 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

---

**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.
**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.
somatic cells surrounding the germ cells in XX gonads immediately after initiation of ovarian differentiation and is maintained in the granulosacells 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 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.

**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.
encoded by two different genes. Tissue distribution analysis revealed that both Cyp19α and Cyp19β were expressed in the gonads and spleen, whereas Cyp19β 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 Cyp19β 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, Cyp19α was not detected in the brain, similar to the situation found in zebrafish (Kishida & Callard 2001). In line with our findings, Cyp19α was also reported to be expressed in the spleen of tilapia (Chang et al. 2005). Spleen is not a steroidogenic tissue. Nevertheless, aromatase and P450scc (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.

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.

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 Dmrt1α and Dmrt1β 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, Cyp19α expression in the gonads and Cyp19β 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...
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. 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, E2 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, E2 treatment up-regulated Cyp19b in the brain and Cyp19a in the gonad. However, E2 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 E2 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 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.

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 ± S.E.M. from five individual fishes. (**p<0.01 as compared with the respective control by one-way ANOVA). C, control; E2, 17β-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. 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
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 F, 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-, and F + TAM-treated fish 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.

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.
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 causal 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 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 have key roles in Southern catfish sex differentiation.
Cyp19a, and Cyp19b are important genes implicated in the sex differentiation of Southern catfish.

Acknowledgements

We are grateful to Professor Y M Tang for the help in cloning the Cyp19a and Cyp19b cDNAs. We thank the Novartis Company in Switzerland for providing the pure powder drug of fadrozole for our studies. This research was funded by grants from the Leading Program of State Education Ministry (No. 2004-104161), the Chongqing Natural Science Foundation (No. CSTC2004BB8450), and the Free Exploration Fund of the Key Laboratory of Eco-environments in the Three Gorges Reservoir Region (Ministry of Education); (No. 124470-20500312). We also thank The Chinese University of Hong Kong for the provision of Direct Grants. The sequences reported in this paper have been deposited in the GenBank database at NCBI and are assigned the following accession nos: EF015487, EF015488, EF015396, AAP83133, and AAP83132. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Chang XT, Kobayashi T, Kajura H, Nakamura M & Nagahama Y 1997 Isolation and characterization of the DNA encoding the tilapia (Oreochromis niloticus) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein, and enzyme activity in ovarian follicles during oogenesis. Journal of Molecular Endocrinology 18 57–66.


Fenske M & Segner H 2004 Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquatic Toxicology 67 105–126.


Guan G, Kobayashi T & Nagahama Y 2000 Sexually dimorphic expression of two types of DM (Doublesex/Mab-3)–domain genes in a teleost fish, the Tilapia (Oreochromis niloticus). Biochemical and Biophysical Research Communications 272 662–666.

Guiguen Y, Baroller JF, Ricordel MJ, Ieki K, Mcmeel OM, Martin SA & Foster A 1999 Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (Oncorhynchus mykiss) and a tilapia (Oreochromis niloticus). Molecular Reproduction and Development 54 154–162.


Kishida M & Callard GV 2001 Distinct cytochrome P450 aromatase isoforms in zebrasfish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology 142 740–750.


Zhang YB & Gui JF 2004 Molecular characterization and IFN signal pathway analysis of Carassius auratus CaSTAT1 identified from the cultured cells in response to virus infection. Developmental and Comparative Immunology 28 211–227.


Received in final form 4 April 2007
Accepted 10 April 2007
Made available online as an Accepted Preprint
16 April 2007

www.endocrinology-journals.org