Molecular cloning and analysis of gonadal expression of Foxl2 in the rice-field eel Monopterus albus

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We isolated the complete Foxl2 (Foxl2a) cDNA from the Monopterus albus ovary. An alignment of known Foxl2 amino-acid sequences confirmed the conservation of the Foxl2 open reading frame, especially the forkhead domain and C-terminal region. The expression of Foxl2 was detected in the brain, eyes, and gonads. A high level of Foxl2 expression in the ovary before sex reversal, but its transcripts decreased sharply when the gonad developed into the ovotestis and testis. The correlation between the Foxl2 expression and the process of sex development revealed the important function of Foxl2 during the sex reversal of M. albus.

Immunohistochemical analysis showed that Foxl2 was expressed abundantly in granulosa cells and in the interstitial cells of the ovotestis and testis. These results suggest that Foxl2 plays a pivotal role in the development and maintenance of ovarian function. Foxl2 may be also involved in the early development of testis and the development of ocular structures of M. albus.

Forkhead transcription factors play a critical role in the regulation of cellular proliferation and differentiation. They are also involved in several other biological processes, including tissue development, establishment of the body axis, metabolic processes, and the determination and differentiation of cell types. In many cases, forkhead transcription factors are also responsible for various processes during embryonic development and adult metabolism.

Forkhead box L2 (Foxl2) is a member of the forkhead family of transcription factors, characterized by a conserved 100-amino-acid domain called "forkhead box". Although the Foxl2 is highly conserved across divergent taxonomic groups, the C-terminal region is more conserved than the N-terminal region outside the DNA-binding forkhead domain. Mammalian Foxl2 contains a polyalanine tract and other low-complexity repeats absent from the fish sequences. Foxl2 mutation in human leads to the blepharophimosis ptosis epicanthus inversus syndrome (BPES), which is characterized by eyelid malformations and premature ovarian failure (POF). Foxl2 was observed in the developing eyelids and perioptic mesenchyme of mouse. It is reported that males and females of mouse lacking Foxl2 are small and show distinct craniofacial morphology with upper eyelids absent. Mouse Foxl2 has also been detected in the pituitary and developing ovary. Recently, many studies demonstrated that Foxl2 is a putative transcription factor in the early development of the female vertebrate gonad and is involved in adult ovarian function. In genetic program, somatic testis determination was activated in an XX gonads mouse lacking Foxl2 from meiotic prophase oocytes, implying the pivotal function of Foxl2 to repress the male gene pathway at several stages of female gonadal differentiation. Foxl2 is also involved in the differentiation of granulosa cells of gonad and the maintenance of ovarian function, as well as the transcriptional regulation of other genes during gonadal differentiation in fish.

Estrogens have important roles in sexual differentiation and sex changes in fish. Aromatase, encoded by cyp19a1a, is responsible for 17β-estradiol (E2) synthesis by catalyzing the conversion of androgens to estrogens. Aromatase and Foxl2 co-localize in the adult ovaries of medaka (Oryzias latipes) and Japanese flounder (Paralichthys olivaceus). The expression profile of Foxl2 also correlates strongly with aromatase activities in the gonads during the sexual differentiation of the chicken (Gallus gallus). These results suggest that Foxl2 is related to the sexual differentiation of fish and other vertebrates through the transcriptional regulation of the cyp19a1a gene.

Rice-field eel Monopterus albus, which belongs to the family Synbranchidae in the order Synbranchiformes (Neoteleostei, Teleostei, Vertebrata), is a hermaphroditic protogynous freshwater fish species that undergoes...
sexual reversal from a functional female to a male\cite{14}. Some genes related to sex determination and differentiation have been identified in *M. albus*, including *cyp19a1a*, *Sox9*, *Dmrt1*, and *Jnk1*\cite{15}. The complete genomic sequences of Fox2 is available for the pufferfish (*Takifugu rubripes*), green spotted puffer (*Tetraodon nigroviridis*), and zebrafish (*Danio rerio*)\cite{16}. Given the detections of Fox2 transcripts in ovary, it is probably involved in ovarian development in mammals\cite{17,18,19}, birds\cite{20}, and teleosts\cite{16,21}. Although there are many studies which investigated the roles of other sex-related genes in *M. albus*, few studies of Fox2 function have been reported in this hermaphroditic fish species. To investigate whether Fox2 is involved in ovarian differentiation and development in *M. albus*, as in other vertebrates, we isolated the full-length Fox2 cDNA from *M. albus*, and analyzed its expression pattern and protein localization in the gonads during sex reversal using real-time RT–PCR and immunohistochemistry.

**Results**

**Isolation and characterization of *M. albus* Fox2.** The *M. albus* Fox2 (Fox2a, KC823043) cDNA is 2037 bp long, which comprises a 263-bp 5′-untranslated region (5′-UTR), an 853-bp 3′-untranslated region (3′-UTR) containing two typical poly(A) signals, and an open reading frame (ORF) (921 bp). The ORF encodes a putative 306-amino-acid containing the 100-amino-acid forkhead domain, signals, and an open reading frame (ORF) (921 bp). The similarity of the nucleotide sequence to those of other vertebrates is available for the pufferfish (*Epinephelus merra*), spotted butterfish (*Scatophagus argus*), and zebrafish (*Danio rerio*). Seven different developmental stages of gonads were tested tissues (Fig. 5).

**Expression profile of Fox2 during sex reversal of the gonads.** To investigate the relationship between Fox2 expression and sex reversal in *M. albus*, we analyzed the expression of Fox2 in the seven different developmental stages of gonads using real-time RT–PCR. The highest level of Fox2 was detected in the ovaries, especially in the fourth-stage ovaries (Fig. 3). However, its expression decreased sharp in the first-stage ovotestis (Fig. 4), and decreased continuously in the second- and third-stage ovotestis (Fig. 4), as well as the testis (Fig. 5). No positive signals were observed in the negative control (Fig. 5).

**Localization of Fox2 protein in the gonads.** In the ovary, Fox2 immunoreactivity was detected abundantly in the granulosa cells around the oocytes and immature oocytes, but not in the mature oocytes (Fig. 7A, B). In the testis and ovotestis, Fox2 was barely detected in the entire testis (Fig. 5, F). No positive signals were observed in the negative control (Fig. 7G).

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**Figure 1** | Nucleotide and deduced amino-acid sequences of *M. albus* Fox2. The amino-acid sequence is shown with the standard one-letter code below the nucleotide sequence. The positions of the amino-acid residues are given in parentheses. The two typical polyadenylation signals are boxed.
Discussion

Two Foxl2 paralogs which are named Foxl2a (Foxl2) and Foxl2b (Foxl3) have been reported in some teleost species. They are also identified by blast against the available fish genome database, which is in agreement with the genome duplication event in fish. Foxl2a and Foxl2b in rainbow trout (Oncorhynchus mykiss) were expressed specifically in the ovary, but displayed different temporal expression patterns. However, the mRNA levels of Foxl2 in ovary and Foxl3 in testis point to a strong sexual dimorphism and vary significantly during the reproductive cycle in European sea bass (Dicentrarchus labrax). Unlike above species, only Foxl2 was cloned in the ovary of M. albus in this study. The same phenomena have been reported in some fish species. Due to the complexity and shortage of date in expression pattern of fish Foxl2b (Foxl3), a systematic investigation on Foxl3 need to be carried out.

The C-terminal region and the forkhead domain of Foxl2 are strongly conserved among different vertebrates. The functions of these two regions may have been conserved throughout evolution, whereas the N-terminal region has evolved under weaker conservation pressure. The forkhead domain of Foxl2 is responsible for the nuclear import of this protein, and contains a putative nuclear localization signal (NLS, typically RRRRRMKR) at C-terminus, like other FOX proteins. Homopolymeric runs of amino acids, such as A, G, and P, were present in the mammalian proteins but not in those of non-mammalian vertebrates. A phylogenetic analysis supported the strong evolutionary conservation of Foxl2 in fish and the close genetic relationship between M. albus and E. merra.

Figure 2 | Alignment of the Foxl2 amino-acid sequences of 11 species. Mon, Monopterus albus; Hal, Halichoeres trimaculatus, BA115129.1; Epi, Epinephelus merra, AC62374.1; Dan: Danio rerio, XP_698915.1; Ore, Oreochromis niloticus, AAT36328.1; Ory, Oryzias latipes, BAF42653.1; Onc, Oncorhynchus mykiss, AAS87040.2; Sil, Silurus meridionalis, ABK76309.1; Hom, Homo sapiens, AAK01352.1; Mus, Mus musculus, AAN04088.1; Sus, Sus scrofa, AAQ91845.1.

Figure 3 | Comparison of Foxl2 amino-acid sequence of M. albus with known orthologues. Abbreviations of the species are the same as in the legend to Fig. 2. The filled bar indicates the forkhead domain. The glycine-rich repeats (G), proline repeats (P), and polyalanine tracts (A) are boxed.
Foxl2 was mainly expressed in the brain, eyes, and gonads of *M. albus*, with the highest level of expression in the ovary. The gonadotropin-releasing hormone receptor gene (*Gnrhr*), which contains a site bound by Foxl2, AP-1, and Smads, can be activated by Foxl2 in mouse. The high level of Foxl2 transcript was found in the *M. albus* brain, indicating that the *Gnrhr* may be regulated by Foxl2 on the transcriptional level. Fish Foxl2 is involved in the hypothalamus-pituitary-gonadal axis due to the detection of Foxl2 expression in the hypothalamus, pituitary, and gonad. In *M. albus*, the transcription of Foxl2 was much higher in gonad than that in brain. It is implied that Foxl2 probably executes its functions via the transcriptional regulation of the gonadotropin-releasing hormone-gonadotropin-sex steroid pathway.

The mutation of Foxl2 leads to BPES, characterized by malformations of the eyelid and premature ovarian failure in human. Compared to BPES, mouse lacking Foxl2 were born with open and nectrotizing eyes, coupled with severe eyelid hypoplasia. Foxl2 expression was concentrated in the perioptic mesenchyme. Lower levels of Foxl2 expression were also detected in the lens fibers of the eyes. Consequently, such expression pattern of Foxl2 could be required for the eyelid formation and the development of other ocular structures in mouse. Foxl2 expression in dogfish (*Scyliorhinus canicula*) was firstly detected in the mesenchyme around the eyes and then restricted to the underlying mesenchyme at the outer edges of the developing eyelids. As in other fish, Foxl2 was highly expressed in the eyes of *M. albus*. This expression pattern manifests the conserved functions of Foxl2 among different species. It is implied that Foxl2 is probably involved in the development of other ocular structures because of the apparent absence of eyelids from most fish.

In general, Foxl2 is used to be considered as a good marker of ovarian differentiation because of its high-level expression in the ovary reported in several vertebrates. Although Foxl2 expression has been detected in the adult testis of several species, the levels of Foxl2 in the testis were all significantly lower than that in the ovary in *G. gallus*, the southern catfish (*Silurus meridionalis*), *Onchorhyncus mykiss*, and frog (*Rana rugosa*). On the contrary, a study in protogynous *H. trimaculatus* demonstrated that Foxl2 expression has no sexual dimorphism between the testis and ovary. Foxl2 expression was also detected in testis of *M. albus*, though it was lower in the testis than that in the ovary. Thus, whether Foxl2 is a good marker of ovarian differentiation in sex reversal species or not should be deliberated.

The highest levels of Foxl2 expression were observed in the ovary before sex reversal in *M. albus*. Foxl2 transcript decreased sharply when individuals developed to the intersex stage I (*♀♂*). Foxl2 expression also declined dramatically as the oocytes continued to degenerate, paralleling with the initiation of germ-cell proliferation into spermatogonia (*♂♂II* and *♀♀III*). Then, Foxl2 expression was maintained at a low level in the testis. The correlation between the Foxl2 expression pattern and the process of sex development reveals the crucial function of Foxl2 during the sex reversal of *M. albus*. Foxl2 was highly expressed in brain and ovary during pre-spawning phase implying the important role for this correlate in ovarian recrudescence in catfish (*Clarias gariepinus*). As the same, high expression of Foxl2 was also observed in brain and ovary of *M. albus*, especially in fourth-stage ovaries (*♀IV*). This result suggests that Foxl2 is involved in the ovarian development and maturity. Foxl2 mRNA in the ovary is restricted to the granulosa (follicular) cells surrounding the oocytes in *Oreochromis niloticus* and *O. latipes*. However, no signals have been observed in the oocytes, as the situation in mammals. In this study, an immunohistochemical analysis showed that the Foxl2 protein was observed abundantly in the granulosa cells around the oocytes and immature oocytes, but not in the mature oocytes. This conserved expression patterns suggest that Foxl2 has a basic function in the differentiation of granulosa cells and a crucial role in the maintenance of oocytes. Lower levels of Foxl2 were detected in the developing testis of mouse from 14.5 dpc (day post conception) onward and in the developing epididymis at later stages by in situ hybridization. In the hermaphrodite *H. trimaculatus*, Foxl2 protein was concentrated in the interstitial cells, including in the tubules and Leydig cells of the testis.

**Figure 4** | Phylogenetic tree based on the Foxl2 nucleotide sequences of 11 species. Abbreviations of the species are the same as in the legend to Fig. 2. The numbers at each branch represent the bootstrap values obtained with 1000 replicates.

**Figure 5** | RT–PCR analysis of Foxl2 mRNA in various tissues of *M. albus*. E, eye; M, muscle; Bl, blood; H, heart; Sk, skin; K, kidney; Sp, spleen; L, liver; Br, brain; T, testis; O-T, ovotestis; O, ovary; –, negative control; 18S rRNA, internal control. The figure was cropped, for uncropped figure, see figure S1 & S2, supplementary file.
Foxl2 mRNA was expressed in testis, and its protein was also observed in the interstitial cells of the ovotestis and testis of *M. albus*. It is inferred that the function of Foxl2 in the testis of sex-reversal species could be quite different from other gonochorous teleosts. Foxl2 might also be involved in the early development and maintenance of testis.

It has been reported that Foxl2 regulates the expression of aromatase, the product of *cyp19a1a*[^1]–[^6], which is the key enzyme in the synthesis of estrogen and important for sexual differentiation in fish[^7]–[^11]. Foxl2 in *G. gallus* is involved in the regulation of aromatase transcription during early sexual differentiation[^12]–[^14]. Foxl2 and aromatase in *Oreochromis niloticus*[^15] and *O. latipes*[^16] are co-expressed in some somatic cells located on the ventral side of the XX gonads. This data suggest the important role of Foxl2 in early ovarian differentiation by activating *cyp19a1a* transcription. Earlier studies of *M. albus* demonstrated that *cyp19a1a* was expressed specifically in the brain and gonads, and then its expression declined significantly with the gonadal development[^17]–[^19]. In this study, *M. albus* Foxl2 was expressed in the brain and gonads. It was also co-expressed with *cyp19a1a* during gonadal development. Taken together, the correlation expression pattern between Foxl2 and *cyp19a1a* in ovary and brain indicates that Foxl2 may play an important role in ovarian development.

In conclusion, Foxl2 expression in the gonads is in line with the process of sex development, revealing the important function of Foxl2 during the sex reversal of *M. albus*. Foxl2 protein and Foxl2 mRNA were detected in the ovaries, ovotestis and testis. Foxl2 was

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**Figure 6** | Copies of Foxl2 transcripts in different phases of *M. albus* gonadal development. Ψ, ovaries; Ψ/♂, ovotestis; ♂, testis.

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**Figure 7** | Immunohistochemical analysis of Foxl2 in different phases of *M. albus* gonadal development. (A–F) Immunohistochemical analysis of Foxl2 in the (A and B) ovaries, (C and D) ovotestis, and (E and F) testis. (B), (D), and (F) are enlarged areas of (A), (C), and (E), respectively. The positive antigen was dyed brown with 3',3'-diaminobenzidine (DAB) (arrows). (G–I) Negative controls.
primarily located in granulosa cells, immature oocytes, and interstitial cells. The Foxl2 mRNA was also observed in brain. These results illustrate that Foxl2 is involved in the early development and maintenance of both ovary and testis. In addition, the detection of Foxl2 expression in eyes suggests the possible function of Foxl2 in the development of ocular structures of *M. albus*.

**Methods**

Fish. Wild *M. albus* were purchased from markets in Wuhan, China. The length of the individuals varied from 34 to 54 cm and their bodyweight ranged from 50 to 120 g. They were kept in fresh water for one week before processing. A small segment of the gonads was fixed in Holland–Bouin’s fluid for histological assessment of the sexual status and immunohistochemical analysis. The remainder was frozen with citric acid buffer (0.1 M citric acid and sodium citrate, pH 6.0), incubated in 3% (v/v) H2O2 and 10% (v/v) normal goat serum to block nonspecific binding, and then incubated overnight at 4°C with the primary antibody (diluted 1:100), which is a synthetic peptide corresponding to residues M(M)ASYPEDPEGT(A) of mouse FOXL2 and the host is rabbit. After incubation with the secondary antibody which is labeled by HRP (anti-mouse/rabbit, Maxxin KIT-9901), the sections were exposed to 3’,3’-diaminobenzidine (DAB) and stained with hematoxylin to visualize the nuclei in the gonadal tissues, and then observed under a fluorescence microscope (Eclipse H600L, Nikon). As the negative control, the sections were treated in the same way but with Tris-buffered saline instead of the primary antibody.

** Gonadal histology.** The fixed gonads were dehydrated in a graded series of ethanol and then embedded in paraffin. Sections (5–8 μm thick) were cut and stained with hematoxylin and eosin. The sexual phase of each fish was confirmed by observation under a light microscope.[25]

**RNA extraction.** After the developmental phase of the gonads was determined following our previous study,[28] total RNA was extracted from the gonads (two samples were selected and RNA was extracted in each phase) and from the blood, muscle, skin, liver, eye (whole eye), spleen, intestines, kidney, heart, and brain (whole brain), according to the RNAPlus manufacturer’s recommendations (Takara, Dalian, China). First-strand cDNA was synthesized from 1 μg of total RNA in a 20 μL reaction volume following the manufacturer’s recommendations of PrimeScript® RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara).

**Molecular cloning of Foxl2.** To clone Foxl2 (Fox2a) from *M. albus*, a pair of degenerate primers was designed from the conserved regions of Foxl2 in other fish species. PCR was performed in a 25 μL reaction volume containing: 2 μL of ovari an cDNA as template, 0.5 μL of each 10 mM primer (see Table S1, supplementary file), 12.5 μL of Premix Taq DNA polymerase (Takara), and double-distilled water to a final volume of 25 μL. 3' and 5' ends of the Foxl2 cDNA were obtained according to the manufacturer’s instructions of the SMART RACE Kit (Clontech, USA). Four gene-specific primers (see Table S1, supplementary file) were designed for the rapid amplification of cDNA ends (RACE). The PCR products were subjected to electrophoresis in 1% (w/v) agarose gel and purified using the TIANquick Midi Purification Kit (Tiangen, China). The purified product was recovered and cloned into the PMD-19T vector (Takara), and then sequenced at the Beijing Genomics Institute.

**Sequence and phylogenetic analyses.** Nucleotide sequences were identified with BLASTN at the NCBI web server. We deduced the amino-acid sequence using the BioEdit software and calculated the percentage identity with the MegAlign program. Clustal W was used to construct a multiple alignment of amino-acid sequences. The phylogenetic trees were constructed using the neighbor-joining method in MEGA version 5.05. The credibility of each branch was supported by the bootstrap scores (1000 replicates). All of the sequences used in multiple alignment and phylogenetic analyses were obtained from GenBank.

**Tissue distribution of Foxl2 expression examined with RT–PCR.** To identify the expression pattern of Foxl2 in various *M. albus* tissues, PCR amplification was performed with the gene-specific primers (see Table S1, supplementary file), which were designed based on the nucleotide sequence we cloned (see above). The PCR protocol was: preheating at 95°C for 3 min, followed by 35 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, with a final extension at 72°C for 10 min. The mixture contained 2 μL of 10-fold diluted cDNA in 25 μL PCR reaction volume (as described above). 18S rRNA (EU120033.1) was amplified with 18 PCR cycles as the internal control to calibrate the expression of Foxl2. All PCR products were subjected to agarose gel electrophoresis in 1% (w/v) agarose gel and the bands were visualized by staining with GelRed[26].

**Foxl2 expression pattern during sex reversal of the gonads examined with real-time RT–PCR.** Absolute quantitative real-time RT–PCR experiments were performed in a final volume of 25 μL containing 2 μL of cDNA, 0.5 μL of each 10 mM primer, and 12.5 μL of SYBR® Premix Ex Taq™ II (Perfect Real Time, Takara). The protocol was: 30 s at 95°C, followed by 30 cycles of 95°C for 5 s, 60°C for 45 s, and 72°C for 30 s. The samples were analyzed in triplicate and the fluorescence released from the dye which was monitored by Rotor-Gene Q. A negative control was containing each assay but cDNA. The standard curve was constructed by the serially diluted plasmid containing the target gene. The expression of Foxl2 in the samples was calculated from the standard curve and expressed as copies/ml. Differences of gene expressions in different developmental stages of the gonads were determined with one-way analysis of variance (ANOVA) following by Tukey’s HSD test. The differences were deemed statistically significant if *P* < 0.05.

**Localization of Foxl2 protein in the gonads with immunohistochemistry.** An anti-Foxl2 polyclonal antibody (PA1-802, Thermo, USA) was used to determine the cellular localization of Foxl2 protein in the *M. albus* gonads. Sections were prepared as described above. After paraffin removal and dehydoration, the sections were washed with citric acid buffer (0.1 M citric acid and sodium citrate, pH 6.0), incubated in 3% (v/v) H2O2 and 10% (v/v) normal goat serum to block nonspecific binding, and then incubated overnight at 4°C with the primary antibody (diluted 1:100), which is a synthetic peptide corresponding to residues M(M)ASYPEDPEGT(A) of mouse FOXL2 and the host is rabbit. After incubation with the secondary antibody which is labeled by HRP (anti-mouse/rabbit, Maxxin KIT-9901), the sections were exposed to 3’,3’-diaminobenzidine (DAB) and stained with hematoxylin to visualize the nuclei in the gonadal tissues, and then observed under a fluorescence microscope (Eclipse H600L, Nikon). As the negative control, the sections were treated in the same way but with Tris-buffered saline instead of the primary antibody.
24. Huang, X. et al. Multiple alternative splicing and differential expression of dmr1 during gonad transformation of the rice field eel. *Biol. Reprod.* 73, 1017–1024 (2005).
25. Xiao, Y. M. et al. Contrast expression patterns of INK1 during sex reversal of the rice-field eel. *J. Exp. Zool. Part B.* 314, 242–256 (2010).
26. Crespo, B., Lan-Chow-Wing, O., Rocha, A., Zanuy, S. & Gómez, A. foxl2 and foxl3 are two ancient paralogs that remain fully functional in teleosts. *Gen. Comp. Endocrin.* 194, 81–93 (2013).
27. Jiang, W. et al. Effects of sexual steroids on the expression of foxl2 in *Gobiocypris rarus.* *Comp. Biochem. Phys. B.* 160, 187–193 (2011).
28. Alam, M. A., Kobayashi, Y., Horiguchi, R., Hirai, T. & Nakamura, M. Molecular cloning and quantitative expression of sexually dimorphic markers Dmrt1 and Foxl2 during female-to-male sex change in *Epinephelus merra.* *Gen. Comp. Endocrin.* 157, 75–85 (2008).
29. Coquet, J. et al. Evolution and expression of FOXL2. *J. Med. Genet.* 39, 916–921 (2002).
30. Hancock, W. W. & Özkaynak, E. Three distinct domains contribute to nuclear transport of murine Foxp3. *PloS one* 4, e7890 (2009).
31. Romanelli, M. G., Tato, L., Lorenzi, P. & Morandi, C. Nuclear localization domains in human thyroid transcription factor 2. *BBA-Mol. Cell Res.* 1643, 55–64 (2003).
32. Kobayashi, Y., Horiguchi, R., Nozu, R. & Nakamura, M. Expression and localization of forkhead transcriptional factor 2 (Foxl2) in the gonads of protogynous wrasse, *Halichoeres trimaculatus.* *Biol. Sex Differ.* 1, 1–9 (2010).
33. Wotton, K. R., French, K. E. M. & Shimeld, S. M. The developmental expression of foxl2 in the dogfish *Scyliorhinus canicula.* *Gene Expr. Patterns.* 7, 793–797 (2007).
34. Wang, H., Wu, T., Qin, F., Wang, L. & Wang, Z. Molecular cloning of Foxl2 gene and the effects of endocrine-disrupting chemicals on its mRNA level in rare minnow, *Gobiocypris rarus.* *Fish Physiol. Biochem.* 38, 653–664 (2012).
35. Liu, Z. et al. Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. *J. Endocrinol.* 194, 223–241 (2007).
36. Oshima, Y., Uno, Y., Matsuda, Y., Kobayashi, T. & Nakamura, M. Molecular cloning and gene expression of Fox2l2 in the frog *Rana rugosa.* *Gen. Comp. Endocrin.* 159, 170–177 (2008).
37. Sridevi, P. & Senthilkumaran, B. Cloning and differential expression of FOXL2 during ovarian development and recrudescence of the catfish, *Clarias gariepinus.* *Gen. Comp. Endocrin.* 174, 259–268 (2011).
38. Pannetier, M. et al. Expression studies of the PIS-regulated genes suggest different mechanisms of sex determination within mammals. *Cytogenet. Genome Res.* 101, 199–205 (2003).
39. Fleming, N. I. et al. Aromatase is a direct target of FOXL2: C134W in granulosa cell tumors via a single highly conserved binding site in the ovarian specific promoter. *PloS one* 5, e14389 (2010).
40. Wang, D. S. et al. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with add binding protein/steroidogenic factor 1. *Mol. Endocrinol.* 21, 712–725 (2007).
41. Wu, G. C., Tomy, S., Nakamura, M. & Chang, C. F. Dual roles of cyp19a1a in gonadal sex differentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli.* *Biol. Reprod.* 79, 1111–1120 (2008).

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# Author contributions

Q.H. wrote this manuscript text; R.T., D.L. and Q.H. designed the experiments; W.G. collected material for study; Q.H., W.G. and Y.G. carried out the experiments and analyzed the data. All authors reviewed the manuscript.

# Additional information

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