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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[1].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[2,3]. In many cases, forkhead transcription factors are also responsible for various processes during embryonic development and adult metabolism[2].

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"[4]. Although the Foxl2 is highly conserved across divergent taxonomic groups[5], the C-terminal region is more conserved than the N-terminal region outside the DNA-binding forkhead domain[6]. Mammalian Foxl2 contains a polyalanine tract and other low-complexity repeats absent from the fish sequences[5]. *Foxl2* mutation in human leads to the blepharophimosis ptosis epicanthus inversus syndrome (BPES), which is characterized by eyelid malformations and premature ovarian failure (POF)[7]. *Foxl2* was observed in the developing eyelids and perioptic mesenchyme of mouse[7]. It is reported that males and females of mouse lacking *Foxl2* are small and show distinctive craniofacial morphology with upper eyelids absent[8]. Mouse *Foxl2* has been also detected in the pituitary[9] and developing ovary[10]. 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[6,11,12]. 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[13]. *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[14–16].

Estrogens have important roles in sexual differentiation and sex changes in fish[17,18]. Aromatase, encoded by *cyp19a1a*, is responsible for 17β-estradiol (E2) synthesis by catalyzing the conversion of androgens to estrogens[19]. *Aromatase* and *Foxl2* co-localize in the adult ovaries of medaka (*Oryzias latipes*)[14] and Japanese flounder (*Paralichthys olivaceus*)[16]. The expression profile of *Foxl2* also correlates strongly with aromatase activities in the gonads during the sexual differentiation of the chicken (*Gallus gallus*)[20]. 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[21]. Some genes related to sex determination and differentiation have been identified in *M. albus*, including *cyp19a1a*[22], *Sox9*[23], *Dmrt1*[24], and *Jnk1*[25]. The complete genomic sequences of *Foxl2* is available for the pufferfish (*Takifugu rubripes*), green spotted puffer (*Tetraodon nigroviridis*), and zebrafish (*Danio rerio*)[15]. Given the detections of *Foxl2* transcripts in ovary, it is probably involved in ovarian development in mammal[7,10], birds[20], and teleost[14–16]. Although there are many studies which investigated the roles of other sex-related genes in *M. albus*, few studies of *Foxl2* function have been reported in this hermaphrodite fish species. To investigate whether Foxl2 is involved in ovarian differentiation and development in *M. albus*, as in other vertebrates, we isolated the full-length *Foxl2* 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 Foxl2*. The *M. albus Foxl2* (*Foxl2a*, 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 conserved sequence of the forkhead family (Fig. 1). The similarity of the *M. albus Foxl2* nucleotide sequence to those of other vertebrates was determined with BLASTN at the National Center for Biotechnology Information (NCBI) web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The result exhibited high levels of identity, especially with the teleosts, including the honeycomb grouper (*Epinephelus merra*, 86%), spotted butterfish (*Scatophagus argus*, 85%), three-spot wrasse (*Halichoeres trimaculatus*, 82%), and peacock bass (*Cichla monoculus*, 82%). A high degree of similarity, *E. merra* (95.1%), Nile tilapia (*Oreochromis niloticus*, 94.4%), and *H. trimaculatus* (94.1%), was also apparent when the percentage identities of the corresponding amino-acid sequences were calculated with Clustal W (Fig. 2). Except the forkhead domain, which displayed almost complete conservation among these fish species, the C-terminal region of Foxl2 was more strongly conserved than the N-terminal region. Like the Foxl2 proteins of other fishes, there is no polyalanine tract or proline or glycine repeat in the *M. albus* (Fig. 3). A phylogenetic tree was constructed with 11 *Foxl2* nucleotide sequences, which clustered into two separate branches when mammalian *Foxl2* were used as the outgroup. *M. albus Foxl2* clusters with *E. merra* displayed the close relationship between them, with 74 bootstrap support (Fig. 4).

Tissue distribution of *Foxl2* expression. RT–PCR showed that *Foxl2* was mainly expressed in the gonads. The level in the ovary was much higher than that in the testis and ovotestis, and there was no obvious difference between the testis and ovotestis. High levels of *Foxl2* expression were also observed in the whole eyes and brain. However, the expression of *Foxl2* was barely detected in the other tested tissues (Fig. 5).

Expression profile of *Foxl2* during sex reversal of the gonads. To investigate the relationship between *Foxl2* expression and sex reversal in *M. albus*, we analyzed the expression of *Foxl2* in the seven different developmental stages of gonads using real-time RT–PCR. The highest level of *Foxl2* was detected in the ovaries, especially in the fourth-stage ovaries (♀IV). However, its expression decreased sharply in the first-stage ovotestis (♀/♂I), and decreased continuously in the second- and third-stage ovotestis (♀/♂II and ♀/♂III), as well as the testis (Fig. 6).

Localization of Foxl2 protein in the gonads. In the ovary, Foxl2 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 ovotestis, positive signals were observed in each type of cell, especially in the immature oocytes and interstitial cells (Fig. 7C, D). Foxl2 expression was also detected in the interstitial cells in testes, but not in sperm (Fig. 7E, F). No positive signals were observed in the negative control (Fig. 7G–I).

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1                                                                                                   GT CAC TTA CAC ACA ACT TGG GTT GCG CCT GCA TCA GAG ACC AGT TTA   47
48    GGA AAA TCT TCG TTG AAG AGA GAA GTG GTA TTT GTG CAT GTC AGC TAC CGA GCT GCA AAA TAA ACG TTT CTG TGA ACG CAG ATT GGA AGA   137
138   CAG CTT TCA TTC GCG GTG CAT CCT GAA GTT CTA CAA CAG ATC ATT TTG CTC ATC GAG GTT ACA AGC GCC TTT AAC ACT TCT GCT TGT TGA   227
228   CGT TTG GAC TGA ACT TGT TTT GTT TTG GTG TGC GCA ATG ATG GCA TCT TAC CAA AGC TTT GAG GAT GAC GCA ATG ACC TTA ATG ATC CAT   317
(1)                                                                 M   M   A   S   Y   Q   S   F   E   D   D   A   M   T   L   M   I   H   (17)
318   GAC AAC AAC ACG AAG AAG GAG AAA GAG CGA CCC AAA GAG GAG CCG GTC GAG GAG AAA GTT TCT GAG AAG TCG GAC CCG ACC CAG AAA CCA   407
(18)  D   N   N   T   K   K   E   K   E   R   P   K   E   E   P   V   E   E   K   V   S   E   K   S   D   P   T   Q   K   P   (47)
408   CCT TAC TCC TAT GTT GCT CTC ATT GCC ATG GCC ATT CGG GAA AGC TCA GAG AAG CGC CTC ACT CTC TCT GGT ATT TAC CAG TAT ATA ATC   497
(48)  P   Y   S   Y   V   A   L   I   A   M   A   I   R   E   S   S   E   K   R   L   T   L   S   G   I   Y   Q   Y   I   I   (77)
498   AGC AAG TTT CCC TTC TAT GAA AAA AAT AAA AAA GGT TGG CAG AAC AGC ATC AGA CAC AAC CTC AGT CTC AAC GAA TGC TTC ATT AAG GTC   587
(78)  S   K   F   P   F   Y   E   K   N   K   K   G   W   Q   N   S   I   R   H   N   L   S   L   N   E   C   F   I   K   V   (107)
588   CCG CGT GAA GGC GGC GGG GAG AGA AAG GGG AAT TAC TGG ACT CTT GAC CCA GCC TGC GAG GAC ATG TTT GAG AAG GGG AAC TAC AGG AGA   677
(108) P   R   E   G   G   G   E   R   K   G   N   Y   W   T   L   D   P   A   C   E   D   M   F   E   K   G   N   Y   R   R   (137)
678   CGC CGC AGA ATG AAG CGG CCG TTC AGA CCT CCA CCT TCG CAC TTC CAG CCG GGT AAG TCC TTG TTC GGA GGA GAC GGC TAT GGC TAC TTG   767
(138) R   R   R   M   K   R   P   F   R   P   P   P   S   H   F   Q   P   G   K   S   L   F   G   G   D   G   Y   G   Y   L   (167)
768   TCC CCA CCC AAG TAC CTG CAG TCT AGC TTC ATG AAC AAC TCT TGG TCG CTA GGC CAA CCG CCT GCT TCG ATG TCC TAC ACG TCC TGT CAG   857
(168) S   P   P   K   Y   L   Q   S   S   F   M   N   N   S   W   S   L   G   Q   P   P   A   S   M   S   Y   T   S   C   Q   (197)
858   ATG GCC AGC GGC AAC GTA AGT CCA GTG AAC GTG AAG GGG TTG TCA GCC CCA TCA TCT TAT AAC CCA TAC TCC CGG GTG CAG AGC ATG GCG   947
(198) M   A   S   G   N   V   S   P   V   N   V   K   G   L   S   A   P   S   S   Y   N   P   Y   S   R   V   Q   S   M   A   (227)
948   CTC CCC AGT ATG GTG AAC TCT TAC AAT GGT ATG AGT CAC CAT CAC CAT CCA GCG CAT CCC CAC CAT GCC CAG CAG CTG AGC CCG GCC ACC   1037
(228) L   P   S   M   V   N   S   Y   N   G   M   S   H   H   H   H   P   A   H   P   H   H   A   Q   Q   L   S   P   A   T   (257)
1038  GCG GCA CCG CCC CCG GTC TCC TCC AGT AAC GGA GCG GGC CTT CAG TTC GCT TGC TCC CGC CAG CCC GCA GAA CTC TCC ATG ATG CAT TGC   1127
(258) A   A   P   P   P   V   S   S   S   N   G   A   G   L   Q   F   A   C   S   R   Q   P   A   E   L   S   M   M   H   C   (287)
1128  TCT TAC TGG GAA CAC GAG ACC AAA CAC TCG GCG TTA CAC ACG AGG ATT GAT ATT TAG AGT TTA CAA GTG GTC TGT GTG AAG TGA GGA CTC   1217
(288) S   Y   W   E   H   E   T   K   H   S   A   L   H   T   R   I   D   I   *                                               (307)
1218  TAA GAG TCA GAG GTA ATT CCT GTG ATT TCA GAA CAG AAA GAA GAC TGT TTT GAA GAG ACG ATT CTG ATT AGC TGT TTG GGT CCA GCT GAC   1307
1308  ATC CAT AAC TGC AAA TGA GGA GTA TGC GGG AGT TGA TGC TGA GAA AGT TTT ACA AAT TGG AGA CGG GAG GTG AAG AAG TCA TCA GGA CAC   1397
1398  ATC AGG TAG CAT AAC CCC TGG GAG CGA TTT GTT GCG CCT GTT ACG CTT GGA CTG ATG GTC ATA ACT TGG TAT TGT AGT CAA ACT GTT CCT   1487
1488  AAA CTT TTC ACT TTC ACG AGG ACA GAC ATT ACA CCT TAG CTG TGT CTA ACT GGT ATG CGT AAT GAT TTC TCT CAA CTT TAC GCA CGG CTT   1577
1578  ATC CTA ACT ATT GCC AAA TGA AAC ATA AAC ATA TGA TGC CCT AAT TAA GTC AAT ACT TTA ATA TGT AAT TAG TCA GAG CTG TTT TTG GCA   1667
1668  TTC TCG GTA ACA TGC ACT GAG CAA GGC CTG CAT GGT GAA ACC TCA CCT CAT CTC ATG TTT GTG AAT CTT GTT AGT GTA AAA AAA AGT GAC   1757
1758  AGT GTG GGT AGA GAT CCA TCA AAC TGC CAA AAA TAC AAT TCA GGG TTT TTC ATA TTT CTT GAT CCC ATA TGT ATG TAT GAT ATA GTT TCC   1847
1848  AGT ATG ACG CCA GCC AGT GCT TGG TTT TGT TAA TCT TTC TAA TTA TTA TAA TTT TTT TTT AAA TAA ACG TGG TTA GCT ATA TTG TCT CAT   1937
1938  TCA TGT TGG GGC CAT GTT TCA TTT TAA TTA GCA CTT TTC CCT CTT TTC TAG GTT TGT GAA TTT CTT AAT AAA CTT CTA TTC GAA AAA AAA   2027
2028  AAA AAA AAA A                                                                                                         2037
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Figure 1 | Nucleotide and deduced amino-acid sequences of *M. albus* Foxl2. 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.

Identity (%)

	1	2	3	4	5	6	7	8	9	10	11		
1		94.1	95.1	82.4	94.4	94.1	91.1	80.9	70.0	69.4	69.7	1	Mon
2	6.1		97.1	82.0	96.4	95.1	93.1	78.9	72.5	70.9	71.6	2	Hal
3	5.1	3.0		83.0	97.7	96.1	93.4	79.9	71.9	70.3	70.9	3	Epi
4	17.1	17.6	16.3		81.8	81.0	82.0	77.3	67.0	68.6	67.6	4	Dan
5	5.8	3.7	2.3	18.2		96.0	94.1	81.6	69.6	69.3	70.3	5	Ore
6	6.1	5.1	4.0	18.8	4.1		92.5	79.3	70.9	69.3	70.6	6	Ory
7	9.1	6.9	6.5	17.6	6.6	7.6		78.3	70.8	69.5	70.8	7	Onc
8	19.8	22.5	21.2	24.2	21.2	21.6	23.4		62.5	65.2	65.6	8	Sil
9	31.9	29.0	30.0	35.5	29.8	30.4	32.1	39.3		96.3	97.9	9	Hom
10	31.9	30.4	31.4	35.5	31.3	31.9	33.1	39.3	3.0		95.5	10	Mus
11	31.3	28.4	29.4	34.8	29.2	29.8	30.9	38.6	1.9	3.3		11	Sus
	1	2	3	4	5	6	7	8	9	10	11		

Divergence

Figure 2 | Alignment of the Foxl2 amino-acid sequences of 11 species. Mon, *Monopterus albus*; Hal, *Halichoeres trimaculatus*, BAJ15129.1; Epi, *Epinephelus merra*, ACD62374.1; 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.

Discussion

Two *Foxl2* paralogs which are named *Foxl2a* (*Foxl2*) and *Foxl2b* (*Foxl3*) have been reported in some teleost species[5,26]. They are also identified by blast against the available fish genome database, which is in agreement with the genome duplication event in fish[27]. *Foxl2a* and *Foxl2b* in rainbow trout (*Oncorhynchus mykiss*) were expressed specifically in the ovary, but displayed different temporal expression patterns[5]. 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*)[26]. 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[14,15,28]. 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[29]. 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[6,30,31]. 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*.

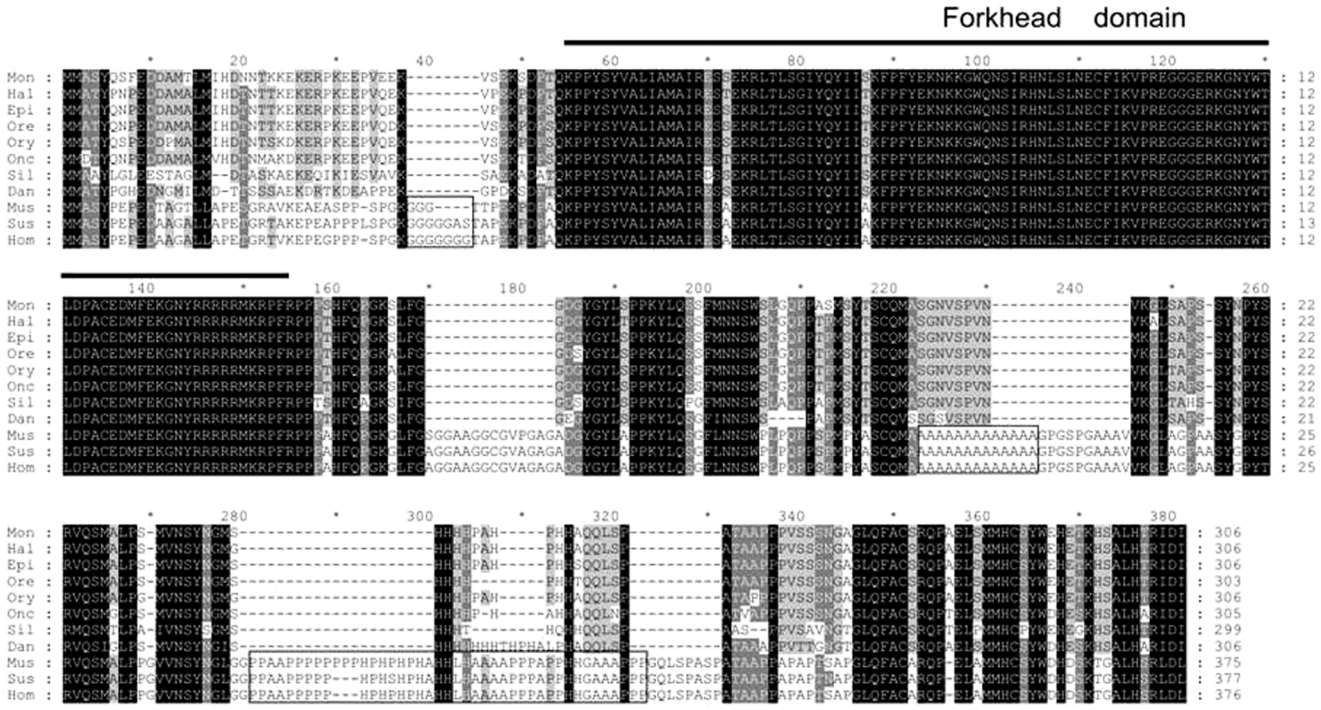

Figure 3 | Comparison of Foxl2 amino-acid sequence of *M. albus* with known orthologues. Abbreviations of the species are the same as in the legend to Fig. 2. The filled bar indicates the forkhead domain. The glycine-rich repeats (G), proline repeats (P), and polyalanine tracts (A) are boxed.

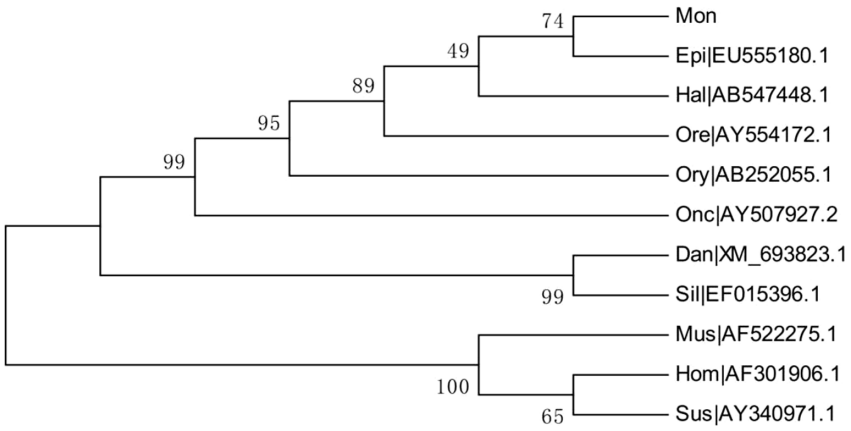

Figure 4 | Phylogenetic tree based on the *Foxl2* nucleotide sequences of 11 species. Abbreviations of the species are the same as in the legend to Fig. 2. The numbers at each branch represent the bootstrap values obtained with 1000 replicates.

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[11]. 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[15,32]. 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[7]. Compared to BPES, mouse lacking *Foxl2* were born with open and necrotizing eyes, coupled with severe eyelid hypoplasia[8]. *Foxl2* expression was concentrated in the perioptic mesenchyme[7]. Lower levels of *Foxl2* expression were also detected in the lens fibers of the eyes[7]. Consequently, such expression pattern of *Foxl2* could be required for the eyelid formation and the development of other ocular structures in mouse[7]. *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[33]. As in other fish[27,32,34], *Foxl2* was highly expressed in the eyes of *M. albus*. This expressive 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[29]. 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*[20], the southern catfish (*Silurus meridionalis*)[35], *Oncorhynchus mykiss*[5], and frog (*Rana rugosa*)[36].On the contrary, a study in protogynous *H. trimaculatus* demonstrated that *Foxl2* expression has no sexual dimorphism between the testis and ovary[32]. *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 (♀/♂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*)[37]. 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*[15] and *O. latipes*[14]. However, no signals have been observed in the oocytes, as the situation in mammals[29,38]. 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[12]. In the hermaphrodite *H. trimaculatus*[32], Foxl2 protein was concentrated in the interstitial cells, including in the tubules and Leydig cells of the testis. A small amount of

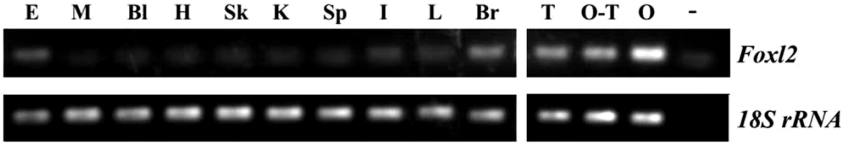

Figure 5 | RT–PCR analysis of *Foxl2* mRNA in various tissues of *M. albus*. E, eye; M, muscle; Bl, blood; H, heart; Sk, skin; K, kidney; Sp, spleen; I, intestine; L, liver; Br, brain; T, testis; O-T, ovotestis; O, ovary; −, negative control; *18S rRNA*, internal control. The figure was cropped, for uncropped figure, see figure S1 &S2, supplementary file.

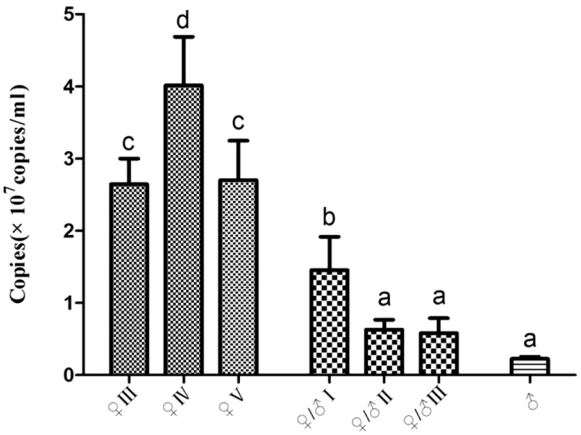

Figure 6 | Copies of *Foxl2* transcripts in different phases of *M. albus* gonadal development. ♀, ovaries; ♀/♂, ovotestis; ♂, testis.

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 teleost. Foxl2 might be also involved in the early development and maintenance of testis.

It has been reported that Foxl2 regulates the expression of aromatase, the product of *cyp19a1a*[39,40], which is the key enzyme in the synthesis of estrogen and important for sexual differentiation in fish[17,41]. Foxl2 in *G. gallus* is involved in the regulation of aromatase transcription during early sexual differentiation[20,42]. *Foxl2* and *aromatase* in *Oreochromis niloticus*[43] and *O. latipes*[14] 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[22,44]. 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

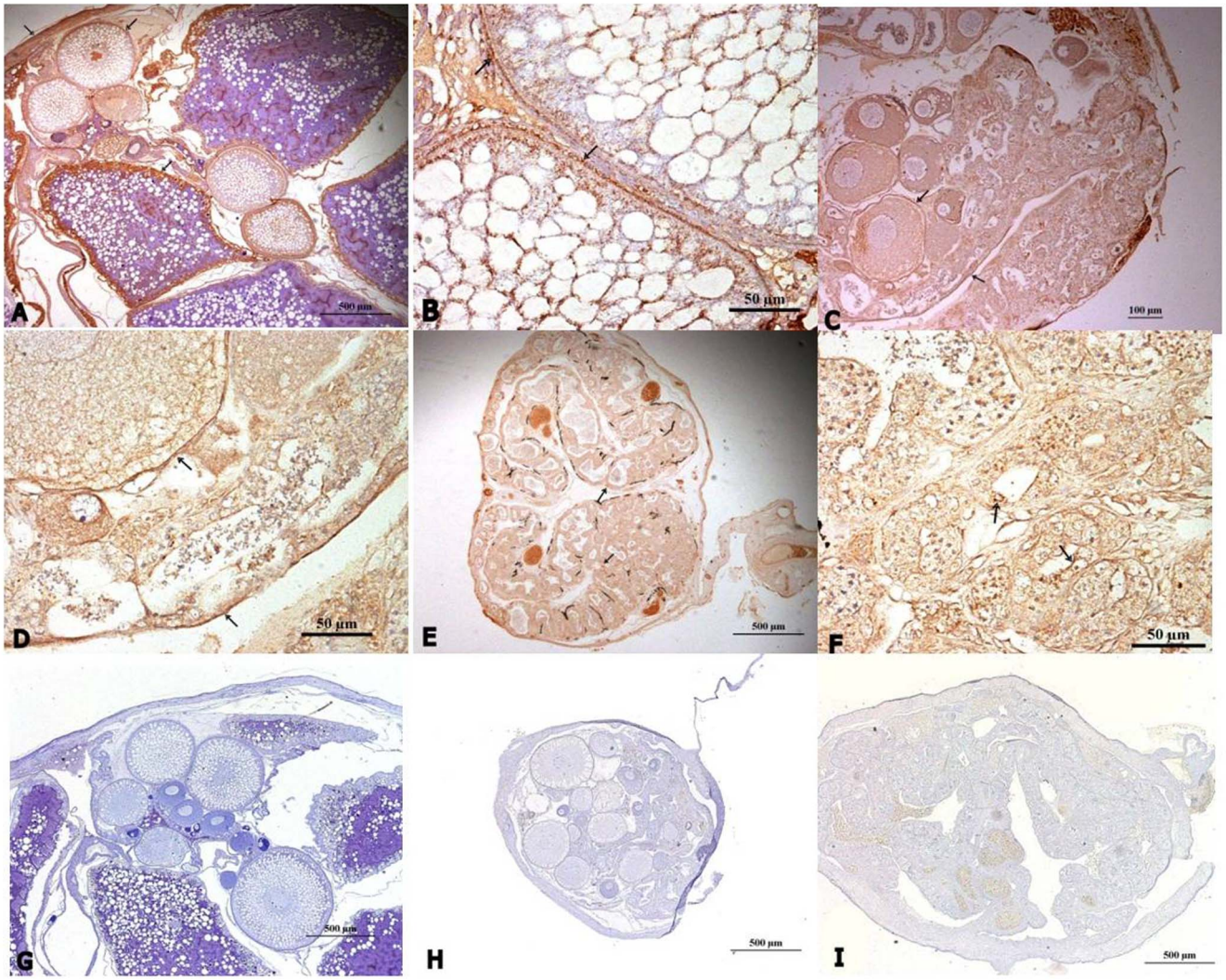

Figure 7 | Immunohistochemical analysis of Foxl2 in different phases of *M. albus* gonadal development. (A–F) Immunohistochemical analysis of Foxl2 in the (A and B) ovaries, (C and D) ovotestis, and (E and F) testis. (B), (D), and (F) are enlarged areas of (A), (C), and (E), respectively. The positive 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 immediately in liquid nitrogen and then stored at −80°C. All experiments were performed with the approval from the Institutional Animal Care and Use Committee of Huazhong Agricultural University (Wuhan, China), strictly according to the guidelines set by this committee for the treatment of animals. The experiments were also performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, promulgated by the Society for the Study of Reproduction.

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[45].

RNA extraction. After the developmental phase of the gonads was determined following our previous study[46], 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 RNAiso Plus 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* (*Foxl2a*) 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 ovarian 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. The 5′ and 3′ 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 electrophoresis in 1% (w/v) agarose gel and the bands were visualized by staining with GelRed™.

***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 at $P < 0.05$.

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 dehydration, the sections were washed with citric acid buffer (0.1 M citric acid and sodium citrate, pH 6.0), incubated in 3% (v/v) H_2O_2 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(1)MASYPEPEDTAGT(14) of mouse FOXL2 and the host is rabbit. After incubation with the secondary antibody which is labeled by HRP (anti-mouse/rabbit, Maixin 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.

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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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