



## OPEN

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

SUBJECT AREAS:

TRANSCRIPTION

MOLECULAR BIOLOGY

Received

14 April 2014

Accepted

13 October 2014

Published

3 November 2014

Correspondence and requests for materials should be addressed to D.P.L. (ldp@mail.hzau.edu.cn)

Qing Hu<sup>1,3,4</sup>, Wei Guo<sup>1,3,4</sup>, Yu Gao<sup>1,3,4</sup>, Rong Tang<sup>1,3,4</sup> & Dapeng Li<sup>1,2,3,4</sup>

<sup>1</sup>College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China, <sup>2</sup>Life Science College, Hunan University of Arts and Science, Changde 415000, China, <sup>3</sup>Freshwater Aquaculture Collaborative Innovation Center of Hubei Province, Wuhan 430070, China, <sup>4</sup>Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, Wuhan 430070, China.

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<sup>1</sup>. 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<sup>2,3</sup>. In many cases, forkhead transcription factors are also responsible for various processes during embryonic development and adult metabolism<sup>2</sup>.

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

Estrogens have important roles in sexual differentiation and sex changes in fish<sup>17,18</sup>. Aromatase, encoded by *cyp19a1a*, is responsible for 17 $\beta$ -estradiol (E2) synthesis by catalyzing the conversion of androgens to estrogens<sup>19</sup>. *Aromatase* and *Foxl2* co-localize in the adult ovaries of medaka (*Oryzias latipes*)<sup>14</sup> and Japanese flounder (*Paralichthys olivaceus*)<sup>16</sup>. The expression profile of *Foxl2* also correlates strongly with aromatase activities in the gonads during the sexual differentiation of the chicken (*Gallus gallus*)<sup>20</sup>. 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<sup>21</sup>. Some genes related to sex determination and differentiation have been identified in *M. albus*, including *cyp19a1a*<sup>22</sup>, *Sox9*<sup>23</sup>, *Dmrt1*<sup>24</sup>, and *Jnk1*<sup>25</sup>. The complete genomic sequences of *Foxl2* is available for the pufferfish (*Takifugu rubripes*), green spotted puffer (*Tetraodon nigroviridis*), and zebrafish (*Danio rerio*)<sup>15</sup>. Given the detections of *Foxl2* transcripts in ovary, it is probably involved in ovarian development in mammal<sup>7,10</sup>, birds<sup>20</sup>, and teleost<sup>14–16</sup>. 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 hermaphroditic 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 butterflyfish (*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).

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	137
138	CAG CTT TCA TTC GCG GTG CAT CCT GAA GTT CTA CAA CAG ATC ATT	227
228	CGT TTG GAC TGA ACT TGT TTT GTT TTG GTG TGC GCA ATG ATG GCA	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	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 G D P T Q K P	(47)
408	CCT TAC TCC TAT GTT GCT CTC ATT GCC ATG GCC ATT CGG GAA AGC	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	587
(78)	S K F P F Y E K N K K G W Q N S C I R H N L S L N E C F I K V	(107)
588	CCG CGT GAA GGC GGG GAG AGA AAG GGG AAT TAC TGG ACT CTT GAC	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 CCG CCG TTC AGA CCT CCA CCT TCG CAC TTC	767
(138)	R 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 TGC CAT CTG CAG TCT AGC TTC ATG AAC AAC TCT	857
(168)	S P P S K Y L Q S S F M N S W S L G Q C 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	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	1037
(228)	L P S M V N S Y N G M S 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 GCG CTT CAG	1127
(258)	A A P P V S S N G A G L Q Q T F C 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	1217
(288)	S Y W E H E T K H S A L H C T R I D I *	(307)
1218	TAA GAG TCA GAG GTA ATT CCT GTG ATT TCA GAA CAG AAA GAA GAC	1307
1308	ATC CAT AAC TGC AAA TGA GGA GTA TGC GGG AGT TGA TGC TGA GAA	1397
1398	ATC AGG TAG CAT AAC CCC TGG GAG CGA TTT GTT GCG CTT GTT ACG	1487
1488	AAA CTT TTC ACT TTC ACG AGG ACA GAC ATT ACA CCT TAG CTG TGT	1577
1578	ATC CTA ACT ATT GCC AAA TGA AAC ATA AAC ATA TGA TGC CCT AAT	1667
1668	TTT TCG GTA ATC TGC ACT GAG CAA GGC CTG CAT GGT GAA ACC TCA	1757
1758	AGT GTG GGT AGA GAT CCA TCA AAC TGC CAA AAA TAC AAT TCA GGG	1847
1848	AGT ATG ACG CCA GCC AGT GCT TGG TTT TGT TAA TCT TTC TAA TTA	1937
1938	TCA TGT TGG GAG CAT GTT TCA TTT TAA TTA GCA CTT TCC CTT TTA	2027
2028	AAA AAA AAA A	2037

**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		
Divergence	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

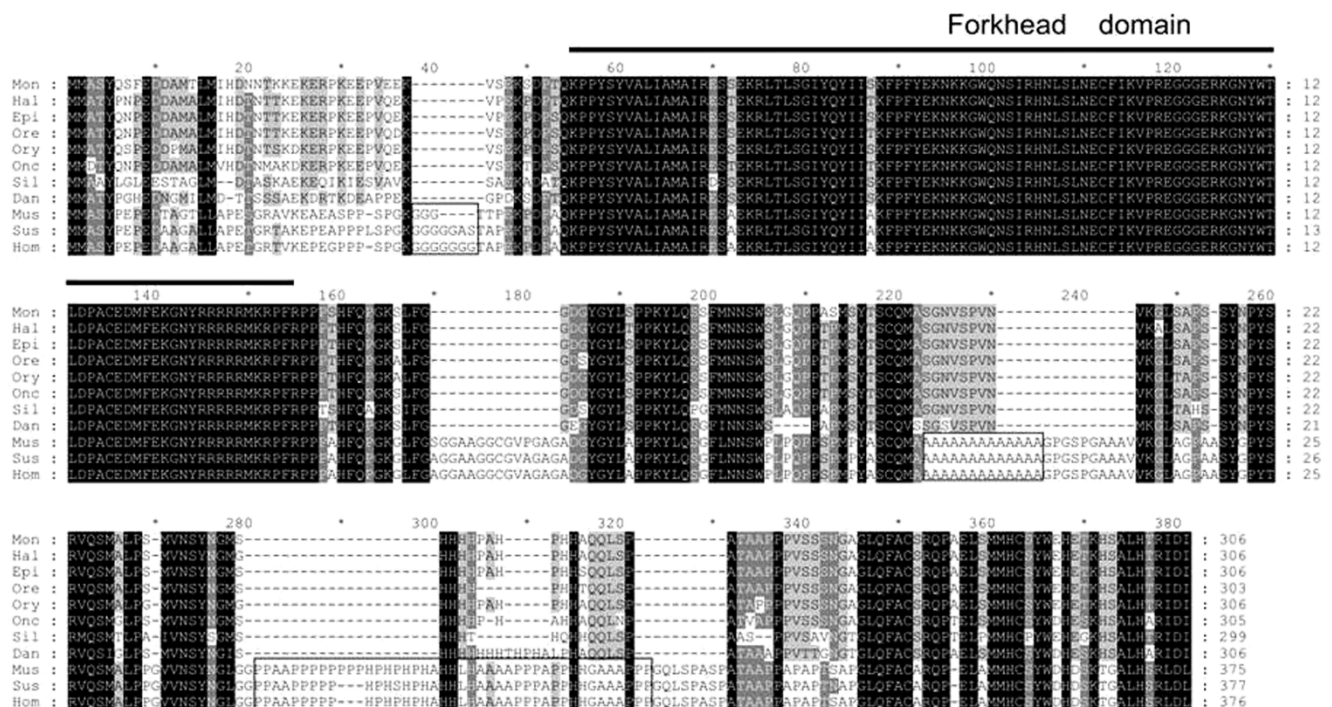
**Figure 2 | Alignment of the Foxl2 amino-acid sequences of 11 species.** Mon, *Monopterus albus*; Hal, *Halichoeres trimaculatus*, BAJ15129.1; Epi, *Epinephelus merra*, ACD62374.1; Dan: *Danio rerio*, XP\_698915.1; Ore, *Oreochromis niloticus*, AAT36328.1; Ory, *Oryzias latipes*, BAF42653.1; Onc, *Oncorhynchus mykiss*, AAS87040.2; Sil, *Silurus meridionalis*, ABK76309.1; Hom, *Homo sapiens*, AAK01352.1; Mus, *Mus musculus*, AAN04088.1; Sus, *Sus scrofa*, AAQ91845.1.

**Discussion**

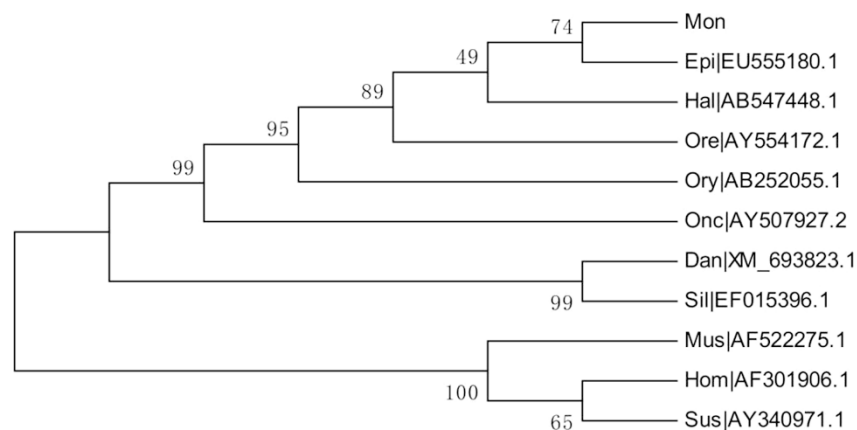
Two *Foxl2* paralogs which are named *Foxl2a* (*Foxl2*) and *Foxl2b* (*Foxl3*) have been reported in some teleost species<sup>5,26</sup>. They are also identified by blast against the available fish genome database, which is in agreement with the genome duplication event in fish<sup>27</sup>. *Foxl2a*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

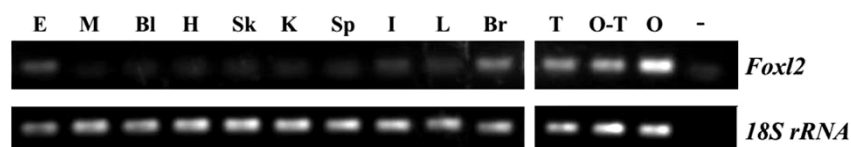
*Foxl2* was mainly expressed in the brain, eyes, and gonads of *M. albus*, with the highest level of expression in the ovary. The gonadotropin-releasing hormone receptor gene (*Gnrhr*), which contains a site bound by Foxl2, AP-1, and Smads, can be activated by Foxl2 in mouse<sup>11</sup>. The high level 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<sup>15,32</sup>. 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<sup>7</sup>. Compared to BPES, mouse lacking *Foxl2* were born with open and necrotizing eyes, coupled with severe eyelid hypoplasia<sup>8</sup>. *Foxl2* expression was concentrated in the perioptic mesenchyme<sup>7</sup>. Lower levels of *Foxl2* expression were also detected in the lens fibers of the eyes<sup>7</sup>. Consequently, such expression pattern of *Foxl2* could be required for the eyelid formation and the development of other ocular structures in mouse<sup>7</sup>. *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<sup>33</sup>. As in other fish<sup>27,32,34</sup>, *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<sup>29</sup>. 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*<sup>20</sup>, the southern catfish (*Silurus meridionalis*)<sup>35</sup>,

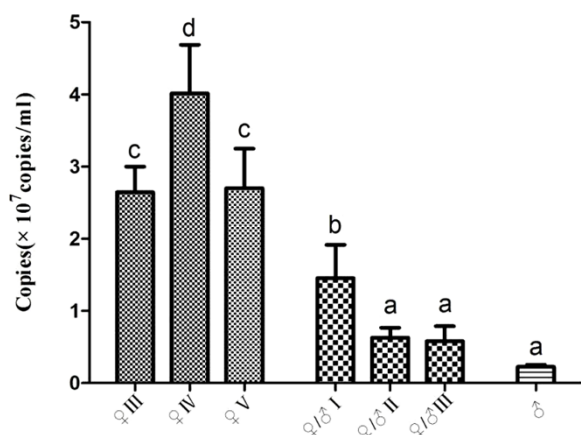
*Oncorhynchus mykiss*<sup>5</sup>, and frog (*Rana rugosa*)<sup>36</sup>. On the contrary, a study in protogynous *H. trimaculatus* demonstrated that *Foxl2* expression has no sexual dimorphism between the testis and ovary<sup>32</sup>. *Foxl2* expression was also detected in testis of *M. albus*, though 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*)<sup>37</sup>. 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*<sup>15</sup> and *O. latipes*<sup>14</sup>. However, no signals have been observed in the oocytes, as the situation in mammals<sup>29,38</sup>. 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<sup>12</sup>. In the hermaphrodite *H. trimaculatus*<sup>32</sup>, 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.





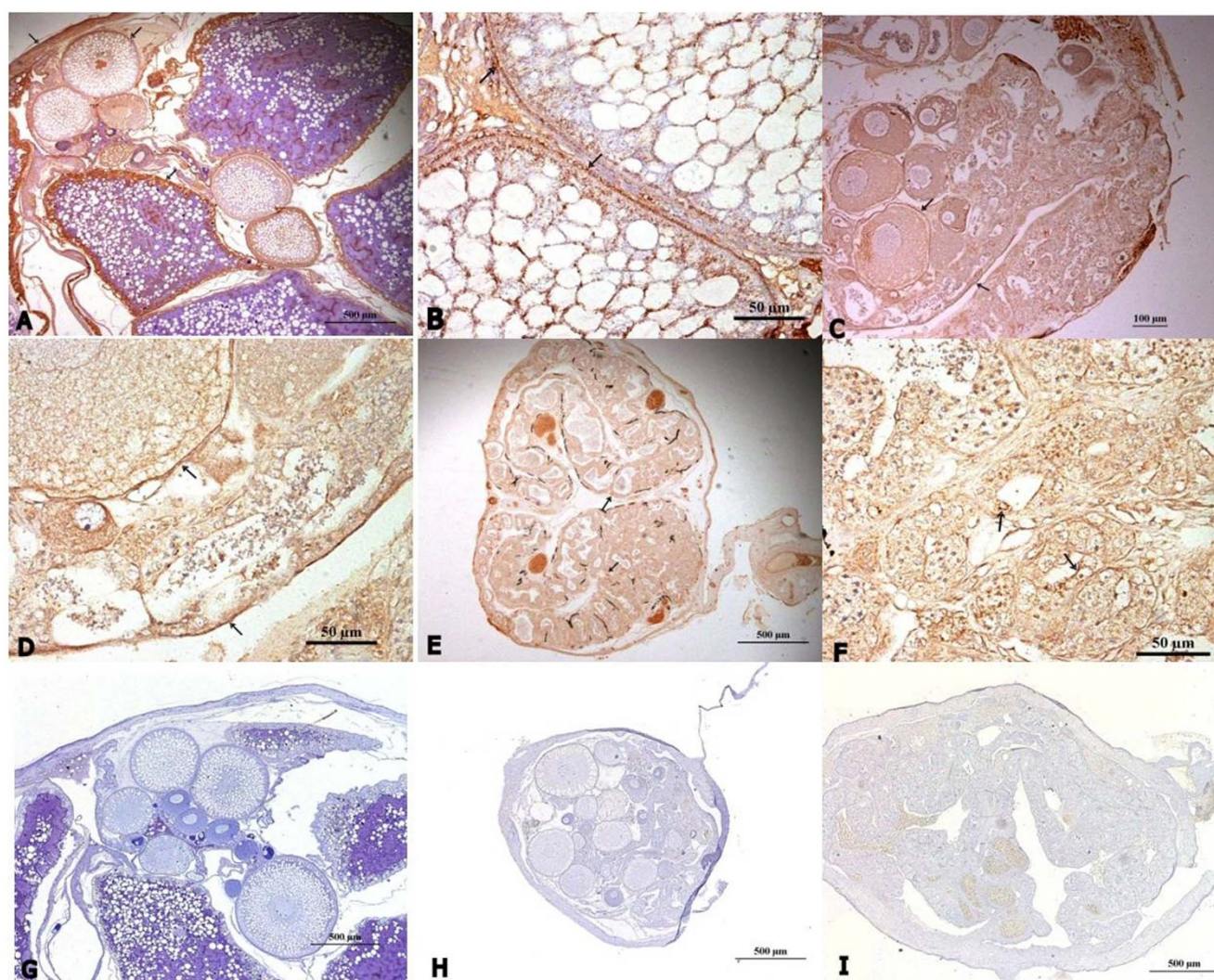
**Figure 6** | Copies of *Foxl2* transcripts in different phases of *M. albus* gonadal development. ♀, ovaries; ♀♂, ovotestis; ♂, testis.

*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*<sup>39,40</sup>, which is the key enzyme in the synthesis of estrogen and important for sexual differentiation in fish<sup>17,41</sup>. *Foxl2* in *G. gallus* is involved in the regulation of aromatase transcription during early sexual differentiation<sup>20,42</sup>. *Foxl2* and *aromatase* in *Oreochromis niloticus*<sup>43</sup> and *O. latipes*<sup>14</sup> 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<sup>22,44</sup>. 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  $-80^{\circ}\text{C}$ . 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  $\mu\text{m}$  thick) were cut and stained with hematoxylin and eosin. The sexual phase of each fish was confirmed by observation under a light microscope<sup>15</sup>.

**RNA extraction.** After the developmental phase of the gonads was determined following our previous study<sup>16</sup>, 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 RNAsiso Plus manufacturer's recommendations (Takara, Dalian, China). First-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA in a 20  $\mu\text{L}$  reaction volume following the manufacturer's recommendations of PrimeScript<sup>®</sup> 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  $\mu\text{L}$  reaction volume containing: 2  $\mu\text{L}$  of ovarian cDNA as template, 0.5  $\mu\text{L}$  of each 10 mM primer (see Table S1, supplementary file), 12.5  $\mu\text{L}$  of Premix Taq DNA polymerase (Takara), and double-distilled water to a final volume of 25  $\mu\text{L}$ . 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  $95^{\circ}\text{C}$  for 3 min, followed by 35 cycles at  $95^{\circ}\text{C}$  for 20 s,  $60^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 20 s, with a final extension at  $72^{\circ}\text{C}$  for 10 min. The mixture contained 2  $\mu\text{L}$  of 10-fold-diluted cDNA in 25  $\mu\text{L}$  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<sup>™</sup>.

***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  $\mu\text{L}$  containing 2  $\mu\text{L}$  of cDNA, 0.5  $\mu\text{L}$  of each 10 mM primer, and 12.5  $\mu\text{L}$  of SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Perfect Real Time, Takara). The protocol was: 30 s at  $95^{\circ}\text{C}$ , followed by 30 cycles of  $95^{\circ}\text{C}$  for 5 s,  $60^{\circ}\text{C}$  for 45 s, and  $72^{\circ}\text{C}$  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)  $\text{H}_2\text{O}_2$  and 10% (v/v) normal goat serum to block nonspecific binding, and then incubated overnight at  $4^{\circ}\text{C}$  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.

- Cunningham, M. A., Zhu, Q., Unterman, T. G. & Hammond, J. M. Follicle-stimulating hormone promotes nuclear exclusion of the forkhead transcription factor FoxO1a via phosphatidylinositol 3-kinase in porcine granulosa cells. *Endocrinology* **144**, 5585–5594 (2003).
- Carlsson, P. & Mahlapuu, M. Forkhead transcription factors: key players in development and metabolism. *Dev. Biol.* **250**, 1–23 (2002).
- Uhlenhaut, N. H. & Treier, M. *Foxl2* function in ovarian development. *Mol. Metab.* **88**, 225–234 (2006).
- Kaestner, K. H., Knöchel, W. & Martínez, D. E. Unified nomenclature for the winged helix/forkhead transcription factors. *Gene Dev.* **14**, 142–146 (2000).
- Baron, D. *et al.* An evolutionary and functional analysis of *FOXL2* in rainbow trout gonad differentiation. *J. Mol. Endocrinol.* **33**, 705–715 (2004).
- Cocquet, J. *et al.* Structure, evolution and expression of the FOXL2 transcription unit. *Cytogenet. Genome Res.* **101**, 206–211 (2003).
- Crisponi, L. *et al.* The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat. Genet.* **27**, 159–166 (2001).
- Uda, M. *et al.* *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Hum. Mol. Genet.* **13**, 1171–1181 (2004).
- Ellsworth, B. S. *et al.* FOXL2 in the pituitary: molecular, genetic, and developmental analysis. *Mol. Endocrinol.* **20**, 2796–2805 (2006).
- Schmidt, D. *et al.* The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development* **131**, 933–942 (2004).
- Ellsworth, B. S. *et al.* The gonadotropin releasing hormone (GnRH) receptor activating sequence (GRAS) is a composite regulatory element that interacts with multiple classes of transcription factors including Smads, AP-1 and a forkhead DNA binding protein. *Mol. Cell. Endocrinol.* **206**, 93–111 (2003).
- Loffler, K. A., Zarkower, D. & Koopman, P. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. *Endocrinology* **144**, 3237–3243 (2003).
- Ottolenghi, C. *et al.* *Foxl2* is required for commitment to ovary differentiation. *Hum. Mol. Genet.* **14**, 2053–2062 (2005).
- Nakamoto, M., Matsuda, M., Wang, D. S., Nagahama, Y. & Shibata, N. Molecular cloning and analysis of gonadal expression of *Foxl2* in the medaka, *Oryzias latipes*. *Biochem. Biophys. Res. Co.* **344**, 353–361 (2006).
- Kobayashi, T. & Nagahama, Y. Molecular cloning and gene expression of *Foxl2* in the Nile tilapia, *Oreochromis niloticus*. *Biochem. Biophys. Res. Co.* **320**, 83–89 (2004).
- Yamaguchi, T., Yamaguchi, S., Hirai, T. & Kitano, T. Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. *Biochem. Biophys. Res. Co.* **359**, 935–940 (2007).
- Guiguen, Y., Fostier, A., Piferrer, F. & Chang, C. F. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *Gen. Comp. Endocr.* **165**, 352–366 (2010).
- Nakamura, M., Bhandari, R. K. & Higa, M. The role estrogens play in sex differentiation and sex changes of fish. *Fish Physiol. Biochem.* **28**, 113–117 (2003).
- Simpson, E. R. *et al.* Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15**, 342–355 (1994).
- Govoroun, M. S. *et al.* Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. *Dev. Dynam.* **231**, 859–870 (2004).
- Liu, C. Rudimentary hermaphroditism in the sybranchoid eel, *Monopterus javanensis*. *Sinensia* **15**, 1–8 (1944).
- Liu, J. F., Guiguen, Y. & Liu, S. J. Aromatase (P450arom) and 11 $\beta$ -hydroxylase (P45011 $\beta$ ) genes are differentially expressed during the sex change process of the protogynous rice field eel, *monopterus albus*. *Fish Physiol. Biochem.* **35**, 511–518 (2009).
- Zhou, R. *et al.* Similar gene structure of two *Sox9a* genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). *Mol. Reprod. Dev.* **66**, 211–217 (2003).



24. Huang, X. *et al.* Multiple alternative splicing and differential expression of *dmrt1* during gonad transformation of the rice field eel. *Biol. Reprod.* **73**, 1017–1024 (2005).
25. Xiao, Y. M. *et al.* Contrast expression patterns of JNK1 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. Endocr.* **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. Endocr.* **157**, 75–85 (2008).
29. Cocquet, 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 *Foxl2* in the frog *Rana rugosa*. *Gen. Comp. Endocr.* **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. Endocr.* **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 ad4 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 schlegelii*. *Biol. Reprod.* **79**, 1111–1120 (2008).
42. Hudson, Q. J., Smith, C. A. & Sinclair, A. H. Aromatase inhibition reduces expression of *FOXL2* in the embryonic chicken ovary. *Dev. Dynam.* **233**, 1052–1055 (2005).
43. Ijiri, S. *et al.* Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. *Biol. Reprod.* **78**, 333–341 (2008).
44. Yu, J. H., Tang, Y. K. & Li, J. L. Cloning, structure, and expression pattern of the P-450 aromatase gene in rice field eel (*Monopterus albus*). *Biochem. Genet.* **46**, 267–280 (2008).
45. Liu, C. & Ku, K. Histological changes in the gonad of *Monopterus* during sex transformation. *Acta. Hydrobiol. Sin.* **2**, 85–109 (1951).
46. Hu, Q., Guo, W., Gao, Y., Tang, R. & Li, D. Reference gene selection for real-time RT-PCR normalization in rice field eel (*Monopterus albus*) during gonad development. *Fish Physiol. Biochem.* DOI 10.1007/s10695-014-9962-3 (2014).

## Acknowledgments

This work was supported by the National Department Public Benefit Research Foundation of China (201003076), the Fundamental Research Funds for the Central Universities (2013PY024), and the National Natural Foundation of China (Project no. 30970529). The authors thank Dr. Jiaoyan Yang, Wei Chi, and Ming Zou for their suggestion on the organization of this manuscript. The authors thank Shimin Lu and Zidong Liu for their technical assistances in this experiment.

## 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

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hu, Q., Guo, W., Gao, Y., Tang, R. & Li, D. Molecular cloning and analysis of gonadal expression of *Foxl2* in the rice-field eel *Monopterus albus*. *Sci. Rep.* **4**, 6884; DOI:10.1038/srep06884 (2014).



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/4.0/>