Foxo3b but not Foxo3a activates cyp19a1a in Epinephelus coioides

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Abstract

FOXO3 has been shown to be a critical transcription factor for folliculogenesis in mammals, while the information on its roles in reproduction of nonmammalian vertebrates remains scarce. In this study, two foxo3 homologs, namely foxo3a and foxo3b, were identified in a teleost, the orange-spotted grouper Epinephelus coioides. foxo3a was mainly expressed in the central nervous system, ovary, and gut whereas foxo3b was expressed ubiquitously in tissues examined. In contrast to the dominant expression of mammalian FOXO3 in germ cells but barely detectable in ovarian follicular cells, immunoreactive Foxo3a and Foxo3b were identified both in the ovarian germ cells and follicular cells. The immunointensities of both Foxo3a and Foxo3b in ovarian follicular cells during vitellogenesis were significantly increased stage-dependently, and co-localized with Cyp19a1a. In the nucleus of ovarian follicular cells, both Foxo3a and Foxo3b immunostaining could be detected at the vitellogenic stages. Transient transfection and EMSA showed that Foxo3a and Foxo3b upregulated cyp19a1a promoter activities in vitro through a conserved Foxo-binding site, with the latter being a more potent activator. However, ChIP analysis showed that only Foxo3b binds to cyp19a1a proximal promoter region containing the conserved Foxo-binding site in the vitellogenic ovary. Taken together, these results suggested that Foxo3a and Foxo3b are involved in the ovarian development possibly through regulating the ovarian germ cells as well as follicular cells, and Foxo3b but not Foxo3a may activate cyp19a1a in the ovarian follicular cells during vitellogenesis in the orange-spotted grouper.

Introduction

Forkhead box-O transcription factors (Foxos) in vertebrates are orthologs of the Daf16 transcription factor which has been shown to play essential roles in longevity, metabolism, and reproduction in Caenorhabditis elegans (Kenyon et al. 1993, Tissenbaum & Ruvkun 1998). In mammals, there are four FOXO genes, FOXO1, 3, 4, and 6, participating in diverse processes including cell proliferation, apoptosis, stress resistance, differentiation, and metabolism (Brunet et al. 1999, Accili & Arden 2004). Recent studies have implicated Foxo3 as a critical transcriptional regulator of ovarian formation and folliculogenesis in mouse (Jagarlamudi & Rajkovic 2012), rat (Reddy et al. 2005,
Liu et al. 2009) and pig (Moniruzzaman & Miyano 2010, Moniruzzaman et al. 2010, Matsuda et al. 2011). Other than mammals, the information on vertebrate Foxo3 is still limited, particularly in relationship with the ovarian development (Diaz et al. 2011).

It has been well documented that steroidogenesis plays key roles in the ovarian development and function. Estradiol at relatively lower levels increased the formation and development of primordial follicles in vitro in hamster, but at higher doses it promotes apoptosis (Wang & Roy 2007), suggesting that regulation of estradiol synthesis is critical in oocyte development. The conversion of estrogens from androgens is catalyzed by an enzyme complex, aromatase, which consists of a flavoprotein NADPH-cytochrome P450 reductase and a cytochrome P450 aromatase (encoded by Cyp19a1 gene) (Kamat et al. 2002). Among the transcription factors possibly involved in the regulation of CYP19A1 gene, AD4BP/SF1 activates CYP19A1 transcription in mammals (Fayard et al. 2004) and in teleosts (Watanabe et al. 1999, Yoshiura et al. 2003). FOXL2, a forkhead transcription factor in subclass L, has also been shown to activate CYP19A1 transcription in goat (Pannetier et al. 2006), and to interact with Ad4bp/Sf1 to upregulate cyp19a1a in tilapia (Wang et al. 2007). Constitutively active Foxo1 mutant suppressed Cyp19a1 expression in rat and mice granulosa cells in vitro (Liu et al. 2009), suggesting that FOXO transcription factors may also be involved in the regulation of Cyp19a1. However, it remains to be established that Foxo transcription factors regulate cyp19a1 expression in vertebrates.

The orange-spotted grouper, Epinephelus coioides, is a protogynous hermaphroditic teleost and a favorite marine food fish in Southeast Asia. It matures as female first and then changes sex around the age of 7 years. Two forms of cyp19a1 gene are present in the orange-spotted grouper, with cyp19a1a predominantly expressed in the gonad while cyp19a1b in the brain (Zhang et al. 2004). Recently, we have isolated and characterized the 5'-flanking region of orange-spotted grouper cyp19a1a, and identified a conserved Foxo-binding site (−99 to −93 relative to the transcription start site) in the proximal promoter region, suggesting that the expression of cyp19a1a may be under the control of Foxo transcription factors (Zhang et al. 2012, 2014). In the present report, two forms of foxo3 genes, namely foxo3a and foxo3b, were isolated and shown to be expressed in the ovarian germ cells as well as follicular cells, and results showed that Foxo3b but not Foxo3a may activate cyp19a1a transcription in ovarian follicular cells during vitellogenesis in the orange-spotted grouper.

Materials and methods

Experimental animals and tissues

The orange-spotted groupers were obtained from Guangdong Daya Bay Fishery Development Center (Huizhou, Guangdong, People’s Republic of China). Fish were killed by decapitation, and tissues were dissected, frozen immediately in liquid nitrogen and stored at −80°C until total RNA or protein extraction. The ovarian development stages were verified by histological observation according to our previous work (Liu et al. 2012, Lu et al. 2014). All procedures and investigations were reviewed and approved by the Animal Research and Ethics Committees of Sun Yat-Sen University, and were performed in accordance with the guidelines of the committee.

Isolation of total RNA

Total RNA was isolated from frozen tissues using TRIzol reagent (Invitrogen) and quantified based on the absorbance at 260 nm. The integrity of RNA was checked with agarose gel electrophoresis.

Cloning of the orange-spotted grouper foxo3a and foxo3b cDNAs

The cDNA was transcribed from total RNA using the RevertAid H Minus First-Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) with primer AP. A PCR product of about 0.45 kb was generated from the ovary cDNA with nested PCR, using the primers EcFoxo-F1 and EcFoxo-R1 for the first round and EcFoxo-F2 and EcFoxo-R1 for the second round of amplification. Eleven clones from the PCR product were sequenced and two different sequences were obtained which correspond to foxo3a and foxo3b of teleosts, respectively. Then the 3’ and 5’ ends of cDNAs were obtained by the RACE method using nested PCR. The details are provided in the Supplementary Materials and methods (see section on supplementary data given at the end of this article).

RT-PCR analysis of foxo3a and foxo3b tissue distribution patterns

Total RNA isolated from tissues was first treated with DNase I (1 U/μL) to remove any genomic DNA contamination. Then, 1 μg total RNA was reverse transcribed with oligo(dT)18 primers using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). The integrity of all
RNA samples was verified by the successful amplification of β-actin (AY510710).

The first-strand reaction (1 μL) was amplified with PCR for each target gene using the TGRADIENT thermocycler (Biometra GmbH, Goettingen, Germany). At least three independent assays were performed to confirm the mRNA distribution patterns in tissues, and the authenticities of the amplicons were confirmed by sequencing. The details are provided in the Supplementary Materials and methods.

Plasmid construction and site-directed mutagenesis

The cDNA fragments encoding Foxo3a and Foxo3b were amplified with primer sets EcFoxo3a-expr-F/EcFoxo3a-expr-R and EcFoxo3b-expr-F/EcFoxo3b-expr-R, and inserted into the pcDNA3.0 vector (Invitrogen) between BamHI and XhoI restriction sites to generate Foxo3a and Foxo3b expression constructs, respectively. These primers are listed in Supplementary Table 1 (see section on supplementary data given at the end of this article). PCR amplification was carried out with Primestar high-fidelity Taq DNA polymerase (Takara Bio).

The serial deletion promoter reporters for the orange-spotted grouper cyp19a1a (JF420889) were constructed in our previous study (Zhang et al. 2012), and these reporters were designated as wild-type (WT) constructs in this study. The putative Foxo-binding site at −99 to −93 was mutated with inverse PCR from 5′-TGTTCAC-3′ to 5′-CAGGGGT-3′ using primer set Eccyp19a1a-Foxo-M-F/Eccyp19a1a-Foxo-M-R. The promoter reporters containing the mutated Foxo-binding site were designated as mutated (MUT) constructs. All the constructs were sequenced in both directions to confirm the authenticity of the sequences.

Cell culture, transient transfection, and luciferase assay

TM4 cells were grown in DMEM/F12 (Invitrogen) supplemented with 2.5% fetal bovine serum (FBS; Gibco) and 5% horse serum (Gibco). COS-7 cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco). Twenty four hours before transfection, cells were plated onto 24-well plates (10⁴ cells/well), and transiently co-transfected with promoter reporter constructs (280 ng/well), an internal control vector pRL-TK (20 ng/well; Promega), and a Foxo3a- or Foxo3b-expression vector (100 ng/well). The Firefly and Renilla luciferase activities were measured 48 h later, and the Firefly luciferase data were corrected for transfection efficiency with Renilla luciferase activity. The details are provided in the Supplementary Materials and methods.

Generation of polyclonal antisera against the orange-spotted grouper Foxo3a and Foxo3b

The cDNA sequences encoding the nonconserved segments of the orange-spotted grouper Foxo3a (aa306 to 451, EcFoxo3a antigen) and Foxo3b (aa308 to 473, EcFoxo3b antigen) were amplified using gene-specific primer sets EcFoxo3a-pET-F/EcFoxo3a-pET-R and EcFoxo3b-pET-F/EcFoxo3b-pET-R, respectively. The primers are listed in Supplementary Table 1. The cDNA fragments were subcloned into the expression vector pET-32a via Ncol and Xhol sites, and expressed in the host E. coli BL21 (DE3) as TRX fusion proteins by the induction of isopropyl β-D-1-thiogalactopyranoside (IPTG). The recombinant EcFoxo3a and EcFoxo3b antigens were gel purified and used to immunize Balb/c mouse as previously reported (Wu et al. 2012).

Western blot analysis

Tissue homogenates (500 μg) were separated onto a 12% SDS–PAGE gel and transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (Millipore) by electroblotting. The membrane was then blocked, immunoreacted with anti-Foxo3a or anti-Foxo3b antisera (1:1000), or anti-ACTB (β-actin) monoclonal antibody (1:500; catalog number: 60008-1-Ig; ProteinTech Group, Inc., Chicago, IL, USA), and processed until exposure to a chemiluminescence substrate (BeyoECL Plus Kit, Beyotime, Jiangsu, China). Details are provided in the Supplementary Materials and methods.

Immunohistochemistry (IHC)

The method was modified from a protocol used in our previous study (Lu et al. 2014). The ovarian sections (4 μm) were deparaffinized, hydrated, and incubated with 3% hydrogen peroxide solution to quench the endogenous peroxidase activity, followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) at 95°C for 15 min and blocking in 0.01 M PBS containing 10% normal goat serum for 30 min at room temperature. Then the sections were incubated sequentially with the primary mouse anti-Foxo3a (1:200) or mouse anti-Foxo3b (1:200) antisera, and the second antibody (HRP-conjugated goat anti-rabbit IgG, 1:500 dilution; Jackson ImmunoResearch Laboratories, Inc.) solution. After rinsing with PBS, the sections were developed with 3,3′-diaminobenzidin (DAB), mounted, examined under Axio Observer Z1 microscope (Carl Zeiss), and digitally photographed.
The immunoreactive levels of Foxo3a and Foxo3b in the ovarian follicular cells were analyzed using the Image Pro Plus software (Media Cybernetics, Inc., PA, USA) in a way similar to our previous study (Lu et al. 2014), and details are provided in the Supplementary Materials and methods.

To confirm the specificity of immunostaining, control sections were incubated with the primary antiserum (in its working dilution) pre-adsorbed with an excess of EcFoxo3a or EcFoxo3b proteins. Additional negative controls included replacement of the primary antiserum with PBS or pre-immune serum and the omission of secondary antibody.

Immunofluorescence

Immunofluorescence was performed as described previously (Lu et al. 2014). Briefly, deparaffinized sections were boiled for 15 min in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval, and blocked in 0.01 M PBS containing 10% normal goat serum for 30 min at room temperature. The blocked sections were then incubated in a primary antiserum mixture of mouse anti-Foxo3a (1:100) or anti-Foxo3b (1:100) with rabbit anti-Cyp19a1a (1:100; Lu et al. 2014) overnight at 4°C. After rinsing with PBS for 10 min, the sections were exposed to the secondary antibody, a mixture of Cy3-labeled goat anti-mouse IgG (H+L) (1:500, catalog number: A0521; Beyotime) and FITC-conjugated goat anti-rabbit IgG (H+L) (1:500; Jackson ImmunoResearch Laboratories Inc.) for 2 h. After washing three times in PBS for 10 min, the sections were counterstained with 5 µg/mL 2-[4-amidinophenyl]-6-indolecarbamidine (DAPI) (Beyotime), a nuclear counterstain, for 20 min at room temperature. After rinsing with PBS for 10 min, the sections were coverslipped using an anti-fade fluorescent mounting medium (Beyotime) and stored in the dark at 4°C. The stained sections were examined under a Zeiss LSM 7 Duo NLO laser scanning confocal microscope (Carl Zeiss). The photographs taken from each photomultiplier were subsequently merged so that the different-colored labels could be visualized simultaneously.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed to examine the binding of Foxo3 to cyp19a1a promoter using the nuclear extracts from COS-7 cells transfected with Foxo3a expression vector pcDNA3.0-EcFoxo3a, Foxo3b expression vector pcDNA3.0-EcFoxo3b, or the empty expression vector pcDNA3.0. The oligonucleotide probes for cyp19a1a promoter were end-labeled with [%32P]dCTP, and incubated with nuclear extracts from COS-7 cells. The reaction mixtures were separated on 5% nondenaturing polyacrylamide gels, and the blots were exposed to a phosphor storage screen (Amersham Biosciences) and visualized with a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, CA, USA). The details are provided in the Supplementary Materials and methods.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with an EZ ChIP assay kit (Millipore) with minor modifications for the tissues of orange-spotted grouper. The sheared chromatin was then used as a template for PCR amplification of the following three regions of cyp19a1a promoters: −246/+67, −1024/+687, and −2226/+1921 bp. The PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide. Details are provided in the Supplementary Materials and methods.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test using SPSS17.0 software (SPSS, Inc.). Significance was set at P < 0.05.

Results

Sequence analysis of the orange-spotted grouper foxo3a and foxo3b

The orange-spotted grouper foxo3a (Genbank accession no. KT588480) and foxo3b (Genbank accession no. KT588481) cDNAs encode putative proteins of 622 and 663 amino acids, respectively. Sequence alignment showed that both Foxo3a and Foxo3b of the orange-spotted grouper contain the conserved putative functional domains, including one N-terminal acidic domain (Acidic), one Forkhead DNA-binding domain, one transactivation domain, two nuclear localization signal (NLS), and one nuclear export signal (NES) (Supplementary Fig. 1, see section of supplementary data given at the end of this article). Three conserved presumptive Akt phosphorylation sites were identified both in Foxo3a and Foxo3b, namely at Thr31, Ser231, and Ser293 for the former, and Thr27, Ser228, and Ser295 for the latter (Supplementary Fig. 1).
The phylogenetic analysis (Fig. 1) showed that the orange-spotted grouper Foxo3a and Foxo3b were grouped with teleost Foxo3a and Foxo3b homologs, respectively, and most closely related to the corresponding forms of tilapia. In the branch of vertebrate Foxo3 proteins, interestingly, teleost, Foxo3b homologs were clustered with tetrapod Foxo3 homologs while teleost Foxo3a homologs were grouped as a separate branch. In addition, the orange-spotted grouper and zebrafish foxo3b genes consist of four exons and three introns which are also conserved in human FOXO3 gene, whereas the orange-spotted grouper and zebrafish foxo3a genes consist of two exons and one intron (Supplementary Fig. 2).

Differential tissue distribution patterns of Foxo3a and Foxo3b

RT-PCR analysis showed that the orange-spotted grouper foxo3b mRNA was present in all tissues examined (Fig. 2).

By contrast, the expression of foxo3a was tissue-specific, with relatively strong signals in the telencephalon, mesencephalon, hypothalamus, cerebellum, pituitary, ovary, foregut, midgut, hindgut, and relatively weak signals in the olfactory bulb, thymus gland, spleen, stomach, heart, and blood, but not or barely detectable in other tissues examined (Fig. 2).

The anti-grouper Foxo3a antiserum and anti-grouper Foxo3b antiserum generated in this study were shown to specifically recognize Foxo3a (Supplementary Fig. 3A) and Foxo3b (Supplementary Fig. 3C), respectively, by western blotting analysis. Foxo3a protein was expressed at higher levels in the brain and ovary, and at a lower level in the spleen, but not in the liver (Fig. 3A). By contrast, Foxo3b protein was expressed in the brain, ovary, spleen, and liver at similar levels (Fig. 3C). The sizes of the proteins detected with western blotting analysis are close to the predicted sizes of Foxo3a (66.3 kDa) and Foxo3b (69.9 kDa), respectively (Fig. 3A and C), and pre-adsorption of the antiserum with an excess of the recombinant full-length Foxo3a or Foxo3b abolished the immunoreactive signals (Fig. 3B and D), further confirming the specificities of the antisera generated.

Expression and co-localization of Foxo3a and Foxo3b with Cyp19a1a in the ovary

Immunohistochemistry was performed to examine the expression of Foxo3a and Foxo3b in the ovary of the orange-spotted grouper during the ovarian development. Foxo3a immunostaining was present in germ cells (at all developmental stages) and in follicular cells. The pre-adsorption of anti-grouper Foxo3a antiserum with the recombinant full-length Foxo3a or Foxo3b abolished the immunoreactive signals (Fig. 3B and D), further confirming the specificities of the antisera generated.

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During the ovarian development, the intensities of Foxo3a immunostaining in the follicular cells were significantly increased from the cortical-alveolus stage to the vitellogenic stage, and then significantly decreased at the mature stage (Fig. 5).

Foxo3b immunostaining was also present in germ cells (at all developmental stages) and in follicular cells. The pre-adsorption of anti-grouper Foxo3b antiserum with the recombinant full-length Foxo3b abolished the immunoreactive signals (Supplementary Fig. 4). At the primary growth stage, cytoplasmic Foxo3b immunostaining was weak in oogonia but strong in oocytes, while nucleic Foxo3b immunostaining was barely detectable in oogonia and faintly detected in oocytes (Fig. 4F). At the cortical-alveolus stage, immunoreactive Foxo3b signals were observed both in the nucleus and cytoplasm of oocytes with great abundances, but faintly in the surrounding follicular cells (Fig. 4G). At the early- and mid-vitellogenic stage, immunoreactive Foxo3b signals were present predominantly in the nucleus of oocytes, and significant immunoreactive Foxo3b signals were also observed in the surrounding follicular cells, particularly at the later stage (Fig. 4H and I). By the mature stage, immunoreactive Foxo3b signals were faint in oocytes, and dramatically decreased in the surrounding follicular cells (Fig. 4J). During the ovarian development, the intensities of Foxo3b immunostaining in the follicular cells was significantly increased from the cortical-alveolus stage to the vitellogenic stage, and then significantly decreased at the mature stage (Fig. 5).

The subcellular localization and co-localization of Foxo3a and Foxo3b with Cyp19a1a in the follicular cells in the ovary from the vitellogenic to mature stages were further examined with immunofluorescence under a confocal microscope (Fig. 6). In the early vitellogenic follicles, nucleic immunoreactive signals for both Foxo3a and Foxo3b were observed in follicular cells (Fig. 6A and C), while in the cytoplasm, immunoreactive Foxo3a and Foxo3b were found to be co-localized with Cyp19a1a signals (Fig. 6B and D). In the mid-vitellogenic follicles, nucleic immunoreactive signals were barely detectable for Foxo3a (Fig. 6E) whereas still prominent for Foxo3b (Fig. 6G), while in the cytoplasm, the immunoreactive signals for Foxo3a, Foxo3b, and Cyp19a1a seemed to be strong and co-localized (Fig. 6F and H). In the mature follicles, immunoreactive signals for Foxo3a, Foxo3b, and Cyp19a1a signals were barely detectable in ovarian follicular cells (Fig. 6I, J, K and L).

At the primary growth stage, immunoreactive Foxo3a signals were present predominantly in the cytoplasm of both oogonia and oocytes, particularly around the nucleus, and also faintly in the nucleus (Fig. 4A). At the cortical-alveolus stage, strong immunoreactive Foxo3a signals were present both in the nucleus and cytoplasm of oocytes, and faint immunoreactive Foxo3a signals were also observed in the surrounding follicular cells (Fig. 4B). At the early- and mid-vitellogenic stage, immunoreactive Foxo3a signals were present predominantly in the nucleus of oocytes, and significant amount of Foxo3a signals were also observed in the surrounding follicular cells, particularly at the later stage (Fig. 4C and D). By the mature stage, immunoreactive Foxo3a signals were faintly detectable in oocytes and dramatically decreased in the surrounding follicular cells (Fig. 4E).
Foxo3a and Foxo3b in ovarian follicular cells

Activation of cyp19a1a promoter by Foxo3a and Foxo3b via the Foxo-binding site in vitro

Foxo3a and Foxo3b significantly upregulated cyp19a1a promoter (Fig. 7A) activities in COS-7 cells (Fig. 7B), with 11.5- and 69-fold increases for the cloned full-length promoter (−2565 to +67 bp) construct, 8.5- and 27-fold increases for the truncated promoter (−1010 to +67 bp) construct, and three- and four-fold increases for the further truncated proximal promoter (−306 to +67 bp) construct. Mutation of the Foxo-binding site (−99 to −93 bp) significantly attenuated or abolished responses of cyp19a1a promoter reporters to Foxo3a and Foxo3b, respectively. Similar results were obtained in TM4 cells for both Foxo3a and Foxo3b, although with lower potencies (Fig. 7C). Moreover, EMSA showed that the orange-spotted grouper Foxo3a and Foxo3b, which were produced in transfected COS-7 cells and confirmed by western blotting analysis (Supplementary Fig. 5), could directly bind the Foxo-binding site (Fig. 8A) of cyp19a1a promoter in vitro (Fig. 8B and C). The specific bands for the protein/DNA complex could be displaced by ×50 (Foxo3b; Fig. 8C) or ×500 (Foxo3a; Supplementary Fig. 6) excessive wild-type...
Figure 6
The sub-cellular localization of immunoreactive Foxo3a (red) and Foxo3b (red) and their co-localization with Cyp19a1a (yellow) in follicular cells surrounding the early vitellogenic (A–D), mid-vitellogenic (E–H), and mature oocytes (I–L). The mouse antiserum against Foxo3a or Foxo3b (1:100) and rabbit antiserum against Cyp19a1a (1:100) were used as primary antisera. The secondary antibody was Cy3-labeled goat-anti-mouse for Foxo3a or Foxo3b, and FITC-labeled goat-anti-rabbit for Cyp19a1a. DAPI was used to stain the nuclei blue. The images were observed and captured under a confocal microscope under the same conditions. The insets within each image are higher magnification of the boxed areas, respectively. The overlapping of the red with the blue or green color generated a purple or yellow color, respectively. Scale bar is 10 µm.
cold competitors, but not by excessive mutated cold competitors (Fig. 8B and C).

**Foxo3b but not Foxo3a binds to cyp19a1a promoter in the vitellogenic ovary**

ChIP was performed to analyze Foxo3a and Foxo3b-binding to cyp19a1a promoter in vivo in the ovary. Foxo3b bound to cyp19a1a proximal promoter in the early and mid-vitellogenic ovary but not the mature ovary (Fig. 9). However, Foxo3a could not bind to cyp19a1a proximal promoter in the ovary at all stages examined (Fig. 9). As cyp19a1a mRNA is expressed in the ovary but not the liver (Zhang et al. 2004), RNA polymerase II was recruited to cyp19a1a proximal promoter in the ovary but not the liver (Fig. 9) as expected, and Foxo3b did not bind to cyp19a1a proximal promoter in the liver accordingly (Fig. 9). Moreover, ChIP did not reveal any binding of either Foxo3a or Foxo3b to cyp19a1a upstream promoter regions, including region −1024/−687 bp and region −2226/−1921 bp (Supplementary Fig. 7).

**Discussion**

Duplicates of foxo3 genes are present in the genomes of teleosts including zebrafish and medaka, as the previously identified foxo5 in fish was later considered to be the ortholog of mammalian FOXO3 (Carter & Brunet 2007), which are presumably due to fish-specific genome duplication (Hoegg et al. 2004). In this study, two homologs of Foxo3, namely foxo3a and foxo3b, were identified in the orange-spotted grouper. Phylogenetic analysis indicated that the orange-spotted grouper Foxo3a and Foxo3b are most closely related to the corresponding forms in teleosts, respectively. Interestingly, Foxo3a of teleost was segregated as an independent branch, whereas Foxo3b is clustered with mammalian FOXO3. Moreover, teleost foxo3b but not foxo3a has a similar genomic structure as that of human FOXO3. These lines of evidence suggest that after duplication, teleost foxo3b is more conserved and probably under more selective pressure than foxo3a during evolution.

The expression of foxo3b mRNA was detected ubiquitously among the tissues examined in the female orange-spotted grouper, whereas the expression of foxo3a mRNA was tissue specific, with predominant expression in the telencephalon, mesencephalon cerebellum, hypothalamus, medulla oblongata, and ovary. The overlapped expression of foxo3a and foxo3b in the brain and ovary of the orange-spotted grouper was further confirmed at the protein level by western blotting analysis. Similarly, the overlapped expression of foxo3a and foxo3b in the brain was also observed in zebrafish, which was suggested to be critical for the development of the nervous system (Peng et al. 2010, Xie et al. 2011), and is in line with neuronal...

In mammals, FOXO3 protein was detected predominantly in the oocytes of primordial and primary follicles in the ovary of mouse (John et al. 2003, Moniruzzaman et al. 2010) and pig (Moniruzzaman et al. 2010), which was implicated in suppressing the primordial oocyte activation (Castrillon et al. 2003, Moniruzzaman et al. 2010). Similarly, our present study also identified strong immunoreactive Foxo3a and Foxo3b signals in germ cells in the ovary of the orange-spotted grouper. In the oogonia, Foxo3a immunostaining was predominantly detected in the cytoplasm, particularly around the nucleic envelope and also faintly in the nucleus, whereas Foxo3b immunostaining was faintly detected in the cytoplasm and barely detectable in the nucleus, suggesting that Foxo3a may play a greater role than Foxo3b in the development of oogonia and/or the maintenance of their stemness in the orange-spotted grouper. In the oocytes from the primary growth stage to the vitellogenic stage, both Foxo3a and Foxo3b immunoreactivities seemed to be strong, particularly within the nucleus. However, at maturation, immunoreactivities of both Foxo3a and Foxo3b were decreased to be faintly detectable. These lines of evidence suggest that oocytes at vitellogenic stages synthesize a large amount of Foxo3a and Foxo3b, which were predominantly localized to the nucleus. In mammals, the nuclear localization of Foxo3 implies an active state of this transcription factor in cell cycle arrest (Reddy et al. 2005). Given that the vitellogenic oocytes in teleosts are arrested at Prophase I of meiosis (Lubzens et al. 2010), it could be hypothesized that the nuclear Foxo3a and Foxo3b in the vitellogenic oocytes of the orange-spotted grouper presumably contribute to the inhibition of Prophase I progression, which awaits further study.

Mammalian FOXO3 proteins have been shown to be undetectable (John et al. 2008, Moniruzzaman et al. 2010, Tarnawa et al. 2013) or barely detectable (Reddy et al. 2005, Matsuda et al. 2011) in the granulosa cells of healthy follicles. By contrast, prominent immunoreactive Foxo3a and Foxo3b signals were identified in follicular (presumably granulosa) cells surrounding the oocytes in the orange-spotted grouper. The immunointensities of both Foxo3a and Foxo3b were increased significantly during vitellogenesis, peaked at the mid-vitellogenic stage, but decreased significantly at the mature stage. These results suggest that both Foxo3a and Foxo3b in follicular cells may also be involved in the ovarian development toward maturation in the orange-spotted grouper.

In vertebrates, cyp19a1 encodes the aromatase, which catalyzes the synthesis of estrogens in granulosa cells and plays important roles in the ovarian development (Guiguen et al. 2010). Although constitutively active Foxo1 mutant suppressed Cyp19a1 expression in rat and mice granulosa cells in vitro (Liu et al. 2009), the involvement of
FOXO transcription factors in the regulation of Cyp19a1a remains to be established yet. Our present study showed that the orange-spotted grouper Foxo3a and Foxo3b could bind the conserved Foxo site, activate cyp19a1a transcription in vitro, and were co-expressed with Cyp19a1a in ovarian follicular cells. However, ChIP analysis showed that Foxo3b but not Foxo3a bound to cyp19a1a proximal promoter region containing the conserved Foxo motif in vitellogenic ovaries, and neither of them could bind to cyp19a1a upstream promoter regions containing putative binding sites for Foxf1, Foxl1, and Foxl2, members of forkhead transcription factors. Furthermore, the expression profile of Foxo3b in ovarian follicular cells seemed to parallel that of Cyp19a1a (Lu et al. 2014) during the ovarian development in the orange-spotted grouper. These lines of evidence suggest that Foxo3b but not Foxo3a in ovarian follicular cells activate cyp19a1a during vitellogenesis, thereby promoting the ovarian development in the orange-spotted grouper. Among some teleosts, particularly in Perciformes, the Foxo-binding site is conserved in cyp19a1a promoters (Zhang et al. 2012, 2014). Therefore, it could be speculated that upregulation of cyp19a1a transcription by Foxo3b in ovarian follicular cells might be conserved in other teleosts.

Consistent with their functional divergence in regulating cyp19a1a transcription in vivo, Foxo3a was shown to be less potent than Foxo3b to activate cyp19a1a promoter both in COS-7 and TM4 cells in vitro. However, immunoreactive Foxo3a in the ovarian follicular cells did exhibit significantly increased expression during vitellogenesis, which suggests that Foxo3a may play roles in the ovarian development possibly mediated by mechanisms different from those of Foxo3b in the orange-spotted grouper. Duplicated genes could be preserved either by neofunctionalization of one of the daughter genes or subfunctionalization of the daughter genes during evolution (Force et al. 1999). Thus it is hypothesized that after duplication, foxo3b retained most of the functions including upregulating cyp19a1a and ubiquitous expression patterns of the ancestral foxo3, whereas foxo3a diverged a lot, acquired new functions in the ovary and other tissues, and preserved possibly through a process of neofunctionalization during evolution. The functions and underlying mechanisms of foxo3a in the orange-spotted grouper are worth further study.

In conclusion, two homologs of foxo3 gene, foxo3a and foxo3b, were identified in the orange-spotted grouper, with differential tissue distribution patterns and the latter being more conserved phylogenetically in sequence and gene structure. Both Foxo3a and Foxo3b proteins were present in the ovarian germ cells as well as follicular cells, and may be involved in the folliculogenesis in the orange-spotted grouper. In the ovarian follicular cells, it is Foxo3b but not Foxo3a that activates cyp19a1a transcription during vitellogenesis in the orange-spotted grouper and possibly other teleosts as well.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0251.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Q L, L Z, and W Z conceived and designed the research; Q L, Y Z, B S, and H L performed experiments; Q L, Y Z, B S, L Z, and W Z analyzed data; Q L, L Z, and W Z wrote the manuscript. All authors approved the final version of the manuscript.

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References

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