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Molecular identification of genes involved in testicular steroid synthesis and characterization of the responses to hormones stimulation in testis of Japanese sea bass (*Lateolabrax japonicas*)

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ABSTRACT

Testicular steroids are critical hormones for the regulation of spermatogenesis in male teleosts and their productions have been reported to be regulated by gonadotropins and gonadotropin-releasing hormone. In the Japanese sea bass (*Lateolabrax japonicas*), the reproductive endocrine, particularly regarding the production and regulation of testicular steroids, are not well understood. For this reason, we first cloned and characterized the response of several key genes regulating the production of testicular steroids and, second, we analyzed the changes of mRNA profiles of these genes during testicular development cycle and in the administration of hCG and GnRHa with corresponding testosterone level in serum, GSI and histological analyses. We succeeded in cloning the full-length cDNAs for the *fushi tarazu* factor-1 (*FTZ-F1*) homologues (*FTZ-F1a* and *FTZ-F1b*), steroidogenic acute regulatory protein (*StAR*) and anti-Müllerian hormone (*AMH*) in Japanese sea bass. Multiple sequence alignment and phylogenetic analysis of these proteins clearly showed that these genes in Japanese sea bass were homologous to those of other piscine species. During the testicular development cycle and hCG/GnRHa administration, quantification of *jsbStAR* transcripts revealed a trend similar to their serum testosterone levels, while a reciprocal relationship was founded between the serum concentrations of testosterone and *jsbAMH* and the links between gonadal expression of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* were also observed. Our results have identified for the first time several key genes involved in the regulation of steroid production and spermatogenesis in the Japanese sea bass testis and these genes are all detected under gonadotropic hormone and gonadotropin-releasing hormone control.

1. Introduction

As in mammals, teleosts steroids play critical roles throughout the reproductive cycle and pituitary gonadotropins (follicle stimulating hormone FSH and luteinizing hormone LH) are the primary mediators of sex steroid synthesis [1]. In males, FSH is required for the initiation of spermatogenesis, whereas the major role of LH is to facilitate gamete maturation and spawning [2]. Binding FSH or LH to their specific cell-surface receptors leads to the production of second messenger molecules which stimulate activity of key steroidogenic enzymes [3]. An important factor regulating the timing and rate of steroidogenesis in mammals appears to be the steroidogenic acute regulatory protein (*StAR*) [4], which may rapidly be synthesized in response to pituitary trophic hormones [5]. In teleosts, studies on *StAR* mainly concentrate on ovarian *StAR* gene expression, i.e., shortfinned eels (*Anguilla australis*) [6], zebrafish (*Danio rerio*) [7], brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) [8] with few information available on the regulation of testicular *StAR* [3,9]. Hence the changes of testicular *StAR* gene expression during spermatogenesis remain to be determined.

Another factor that has recently been characterized as an important contributor to the regulation of sex determination and later gonad development in several teleost species like Japanese eel (*Anguilla japonica*) [10], medaka (*Oryzias latipes*) [11], zebrafish [12], European sea bass (*Dicentrarchus labrax*) [13] is the anti-Müllerian hormone (*AMH*), also known as Müllerian inhibiting substance (*MIS*). It belongs to the transforming growth factor β (TGF-β) superfamily [14]. It is reported that *AMH* negatively modulates the differentiation and function of Leydig cells by

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down-regulating several enzymes involved in the steroidogenic pathway [15,16]. In addition, a reciprocal relationship between the serum concentration of testosterone and MIS has been found in the postnatal male mammal [17–19]. However, the regulatory mechanisms of *AMH* in teleosts are not fully understood yet. Although several other factors (SOX-9, GATA4 and Wilms tumour-1, WT1) have been identified to be essential to *AMH* expression in mammal [20,21], SF-1 (steroidogenic factor 1), a member of the *fushi tarazu* factor 1 (*FTZ-F1*) subfamily of nuclear receptors, is wildly supposed to be crucial in mammalian steroidogenesis by regulating *AMH* expression [20,22].

FTZ-F1 was first found to be an activator of *fushi tarazu* (*FTZ*) in Drosophila [23], afterwards the *FTZ-F1* homologs were renamed NR5A according to the novel nomenclature system [24] which have been divided in two groups (NR5A1 and NR5A2). Mammalian steroidogenic factor 1 (SF-1) and genes homologous to SF-1 are placed in the NR5A1 [25], while The NR5A2 group contains genes coding for proteins linked to regulation of α-fetoprotein [26]. However, in teleosts, the *FTZ-F1* genes display overlaps between the mammalian NR5A1 and 2 expression domains [27–29], showing that the mammalian classification system may not be appropriate to apply on fish [22,30]. Although the involvements of reproduction have been established for different salmonids and zebrafish *FTZ-F1* genes (ff1a–d) [22,27,30–32], the function of *FTZ-F1* in teleost has not been fully elucidated. Studies on medaka and tilapia also demonstrate that *FTZ-F1* is a transcriptional regulator for aromatase expression and activity [33,34]. Thus, in teleosts, *FTZ-F1* may play an important role in regulation of steroidogenic enzyme expression.

Japanese sea bass (*Lateolabrax japonicas*) is an important marine fish which is widely distributed and particularly interested for commercial aquaculture in parts of East Asia countries including China. In China, because of the sharp decline in population size of wild mature Japanese sea bass and the short time of cage cultivation, considering the asynchronous of female and male gonadal development, it is always difficult to get enough mature male fish for artificial breeding when the female fish ovulation, so external hormones are urgently needed to improve the male gonad development. Human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH) analogue-GnRHa have been employed to accelerate gamete maturation in many fish, i.e., rainbow trout [35], coho salmon (*Oncorhynchus kisutch*) [36] and black porgy (*Acanthopagrus schlegeli*) [37], therefore these two hormones were chosen to administrate the testis development in Japanese sea bass. However, the specific effects of hCG and GnRH stimulation on testicular function have not been examined to date in this specie and comprehensive studies on the expression of testicular steroidogenic enzyme genes during spermatogenesis in Japanese sea bass are still needed to be investigated. Thus, the aims of this study are to determine the changes of *StAR*, *AMH* and *FTZ-F1* mRNA expression in Japanese sea bass testes during spermatogenesis and administration of hCG and GnRHa together with the changes in histological and testosterone level in serum.

2. Materials and methods

2.1. Fish treatment and sampling

Seventy-one Japanese sea bass (body weight 671.21 ± 75.25 g and body length 37.78 ± 1.57 cm) were sampled from a commercial fish farm (Qingdao, China) in November 2011 and acclimatized in 16 tanks for 3 days. Fish were reared in natural sea water under controlled conditions (temperature 17.5 ± 0.7 °C; dissolved oxygen = 7.5 ± 0.45 mg/l; 13 h light: 11 h dark cycle; salinity 29.0 ± 0.8‰). Seawater was 2/3 replaced daily. After the adaption period, the fish were divided into three groups randomly, two treatment groups were intraperitoneal injected with GnRHa (des Gly^{10}-[D-Ala^{6}]) and hCG (Ningbo, China) at 3.5 μg/kg body weight and 1000 units/kg body weight, respectively [38,39], the control group was injected with physiological saline solution (PS). Four male fish for each group were anaesthetized with 0.2% MS-222 (Sigma, St. Louis, MO) at 0, 6, 12, 24, and 48 h. The weights of the testes were recorded for computing the GSI, GSI = (gonad weight/[body weight − viscera weight]) × 100 [40]. Then tissues including testis, liver, stomach, gills, heart, spleen, kidney, head kidney, intestine, brain, pituitary and muscle were quickly removed under sterile condition, snap-frozen and stored at −80 °C until analysis.

For the research of the testicular development cycle, six male Japanese sea bass were obtained every month during periods of spawning season (September–December) in 2011, fish were acclimatized 3 days in laboratory and anesthetized with MS-222. All tissues were removed rapidly and kept at −80 °C.

2.2. Histological analysis

The testis were fixed in Bouin's solution for more than 24 h then dehydrated in a graded series of ethanol, embedded in paraffin and cut to 5 μm sections by microtome (LEICA-RM2016), followed by hematoxylin and eosin (H.E.) staining and photographing by light microscopy (Nikon-E200, Japan). The testis developmental stages were determined mainly based on other teleosts [40].

2.3. Radioimmunoassay

Serum levels of testosterone in male Japanese sea bass were measured using Iodine (^{125}I) Radioimmunoassay Kits (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Sino-US joint-venture enterprise), according to the method described by Shi [40]. The coefficients of intra-assay and inter-assay variation were 7.4% and 9.8%, respectively for the assay.

2.4. Total RNA extraction and reverse transcription (RT)

Total RNA was extracted using RNAiso reagent (Takara, Japan) according to the manufacturer's protocol. Briefly, tissues were homogenized in RNAiso, precipitated isopropanol and washed in 75% ethanol. After DNase treatment, the concentration of total RNA were quantified by the Nucleic acid analyzer, Biodropsis BD-1000 (OSTC, China) and a 1.5% agarose gel was applied to detect the integrity. The reverse-transcription of 2 μl total RNA was carried out using M-MLV Reverse Transcription Kit (Promega, USA) and the resulting first strand cDNAs were used as templates.

2.5. Cloning of StAR, AMH and FTZ-F1 from Japanese seabass testis

To obtain the core fragment of steroidogenic acute regulatory protein (*StAR*), anti-müllerian hormone (*AMH*) and *fushi tarazu* factor-1 (*FTZ-F1*) of the Japanese sea bass, three pairs of degenerate primers were designed (Supplementary Table 1) on the CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop.html), according to previously reported homologous gene sequences in teleosts. The PCR was performed in a final volume of 25 μl containing 2.5 μl of 10× reaction buffer, 2 μl of 10 mM dNTP mix, 0.5 μl of 25 μM solution of each primer, 2.5 U of rTaq polymerase (Takara, Japan) and 18.25 μl sterile water. The PCR reaction was carried out with 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at the annealing temperature, and 50 s at 72 °C and followed by an additional 10 min at 72 °C to extend. PCR product was electrophoresed, purified then cloned into pGEM-T vector (Tiangen, China) followed by propagation in *Escherichia coli* DH5α and sequenced to get the nucleotide information. The result of blasting in

NCBI revealed that the cloned fragments shared high homology with genes from other teleosts. In order to obtain their full-length sequences, the RACE (Rapid Amplification of cDNA Ends) method was carried out according to the manufacturer's protocol, with four specific primers (Supplementary Table 1) for each gene were designed based on the nucleotide sequence obtained above.

2.6. Homology analysis

Multi-sequences with deduced amino acid sequences of *StAR*, *AMH* and *FTZ-F1* were gained from NCBI and aligned using CLUSTAL W [41]. MEGA 5.0 software package was applied to construct and analyzed phylogenetic tree using the UPGMA method with 1000 bootstrap trials.

2.7. Tissue-specific expression of jsbStAR, jsbAMH and jsbFTZ-F1 genes

A semi-quantitative PCR was set up and applied to measure transcript abundances of these three genes in different tissues from male Japanese sea bass at stage V. Total RNA (2 μg) of tissues including testis, liver, stomach, pituitary, gills, heart, spleen, kidney, head kidney, intestine, brain, and muscle were isolated as described above. The *18S* rRNA (internal control gene) primers and gene-specific primers were listed in Table 1, as well as the reaction temperatures and length of productions. After separating on 1.5% agarose gels, the PCR products were electrophoresed and analyzed by the software: Chemiluminescent and Fluorescent Imaging System (SAGECREATION, China).

2.8. Real-time quantitative RT-PCR assay

Real-time quantitative PCR (q-PCR) was performed with the SYBR Premix Ex Taq (TAKARA, Japan) on Roche 480 light cycler system to measure the changes of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* gene expression during testicular development cycle and hormone administration. Three pairs of specific primers and internal control gene (*18S*) primers were list in Table 1, The SYBR green assay for every gene was optimized for primer concentration and annealing temperature to obtain apposite standard curve and a single sequence-specific peak in the dissociation curve. Q-PCR amplification was carried out in duplicate along with a no-template control in a total volume of 25 μl: containing 12.5 μl of SYBR Premix, 2 μl of the 1:10 diluted cDNA, 0.5 μl each of forward and reverse primer and 9.5 μl PCR grade water. The thermal cycling parameters were an initial 1 cycle activation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, Tm of each gene for 15 s, 72 °C for 15 s, and a dissociation curve was produced *StAR*ting from 55 °C (+1 °C/30 s) to 95 °C. After the PCR program, $2^{-\Delta\Delta C_T}$ method was used to analysis the expression levels. Samples from the hCG and GnRH-injected groups were expressed relatively to that of the PS-injected group (experimental controls) as fold change and the samples in testicular development cycle were relative to that of stage II.

2.9. Statistics analysis

Statistical analyses were performed using SPSS13.0 (SPSS, Chicago, IL). Statistical differences of genes expression changes during testicular reproduction cycle and hormone-treatments were analyzed by Duncan's multiple range tests and differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Gonadal development

The histological and morphological photomicrographs of Japanese sea bass testes applied in hormone injection experiment were showed in Fig. 1. Typical early period of stage V testes were detected in fish without any treatment. Obviously, seminiferous tubules were filled with some mature spermatozoa, and spermatids can be distinguished evidently (Fig. 1A and B). After injecting the external hCG for 48 h, large quantities of mature spermatozoa were detected in the testis and the development level had been improved significantly (Fig. 1D and E). The changes of GSIs during reproduction cycle were displayed in Fig. 2A, GSIs were low during stages II–IV, and increased significantly during late-spermatogenesis and spermiation, stage V (6.8-fold compared to stage IV, $P < 0.05$). Changes in GSIs of Japanese sea bass testes in hCG/GnRHa administration were shown in Fig. 2B. The average GSI was 2.27 ± 0.15 before injecting external hormones, while it increased markedly to 1.39-fold after injecting hCG for 48 h ($P < 0.05$).

3.2. Changes of serum testosterone levels during testicular development cycle and hCG/GnRHa administration

Changes in serum testosterone levels corresponding with testis developmental stage are showed in Fig. 3A. It remained low in both immature and early-spermatogenesis males, ranging from 34.04 ± 3.70 ng/dl at stageII to 47.91 ± 5.43 ng/dl at stage IV. However, it significantly increased to 112.74 ± 9.28 ng/dl at stage V ($P < 0.05$).

Levels of serum testosterone were increased in response to the in vivo administration of hCG and GnRHa (Fig. 3B). Levels of testosterone in the GnRHa-injected group were approximately 2.5-fold higher than those in the PS-injected group at 24 h (98.90 ± 12.59 ng/dl and 28.55 ± 2.42 ng/dl, respectively, $P < 0.05$). The increase of T levels in the hCG-treated groups was stronger than that in GnRHa group. It showed approximately 5.4-fold, 12.3-fold and 11.2-fold increase over the controls at 12, 24 and 48 h (12 h: 199.14 ± 32.48 ng/dl, 24 h: 343.74 ± 24.25 ng/dl and 48 h: 305.24 ± 29.11 ng/dl, $P < 0.05$).

3.3. Isolation and characterization of FTZ-F1a, FTZ-F1b, StAR and AMH cDNAs from the Japanese sea bass testis

By using the degenerate primers, two clones were identified with sequences similarity to teleost *FTZ-F1*. Further sequence char-

Table 1
Primer sequences used in gene expression analyses (q-PCR and RT-PCR) of *StAR*, *AMH* and *FTZ-F1* from Japanese sea bass.

Gene	Forward primer	Reverse primer	Product size (bp)	PCR efficiency (%)	T_m
StAR	AATGGGGGAGTGGAACCCTAA	AGCGGACGCTGACAAAGTC	137	93	62
AMH	CCGTGCGTATGAGGTGC	GTTGGCGGTGTTTGGAC	136	93	61
FTZ-F1[*]	TGCCTCAAGTTCCTGGTCCT	CGTTTGCTGCGGGTAGTTAC	112	90	62
18S	GCGGTCGGCGTCCAACTTCT	CGAGTGGGGTTCAGCGGGTT	174	108	59

[*] The primers used to analysis tissue distribution and changes of *FTZ-F1* mRNA profile during testicular development cycle and hCG/GnRHa administration were designed in conservative region, region III, of the *jsbFTZ-F1a* and *b*, so the whole *FTZ-F1* was detected in this study.

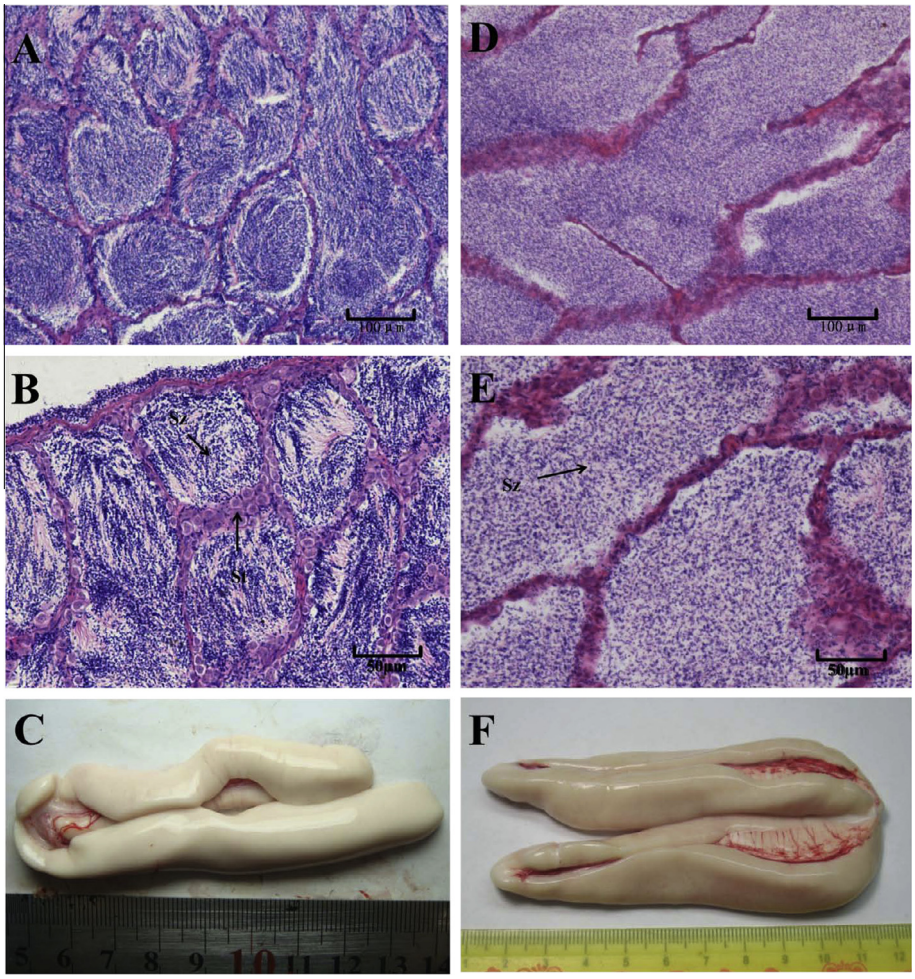


Fig. 1. Histological and morphological photomicrographs of Japanese sea bass testes applied in the hormone administrations. (A and B) The testis in early period of stage V, A: bar = 100 μm, B: bar = 50 μm. (C) The morphology of A and B, the testis before injecting hormone, GSI = 2.65%. (D and E) The testis in last period of stage V, D: bar = 100 μm, E: bar = 50 μm. (F) The morphology of D and E, the testis after injecting hCG for 48 h, GSI = 6.79%. St, spermatid; Sz, spermatozoa.

acterization yielded the full-length cDNA sequence of *jsbFTZ-F1a* and *jsbFTZ-F1b*. The *jsbFTZ-F1a* cDNA resulted in a 1575 bp sequence that contained a 18 bp 5′UTR, a 1557 bp ORF, encoding a predicted protein of 518 amino acids, and the 1499 bp *jsbFTZ-F1b* contained 89 bp 5′UTR, a 1410 bp ORF, encoding a predicted protein of 534 amino acids. The conservative regions of *FTZ-F1a* and *FTZ-F1b*, Regions I, II, III [42] and the *FTZ-F1* box [43], were indicated in Fig. 4A. What's more, comparison of the deduced amino acid sequences with other available sequences evidenced that *jsbFTZ-F1a* possessed high homology with European sea bass *NR5A2* (97%), mangrove rivulus (*Kryptolebias marmoratus*) *FTZ-F1* (96%), black porgy *FTZ-F1a* (96%) and *jsbFTZ-F1b* possessed high homology with European sea bass *NR5A1b* (71%), *jsbFTZ-F1a* (68%) and zebrafish *ff1a* (68%), respectively (Fig. 4B). Phylogenetic analyses of vertebrate *FTZ-F1* proteins clearly showed that there were mainly two separate *FTZ-F1* homologues in Fig 4C. The *jsbFTZ-F1a* was placed in NR5A2 clade, closest related to zebrafish, rainbow trout and mangrove rivulus *FTZ-F1* sequences and the *jsbFTZ-F1b* aligned within NR5A4 clade together with orange-spotted grouper (*Epinephelus akaara*) and European sea bass *FTZ-F1* homologous.

In the case of *jsbStAR* and *jsbAMH*, the 1234 bp *jsbStAR* cDNA contained a 157 bp 5′UTR, an 858 bp open reading frame (ORF) and a 219 bp 3′UTR, encoding a predicted protein of 286 amino acids, and the 2219 bp *jsbAMH* cDNA contained a 70 bp 5′UTR, a 1602 bp ORF and a 547 bp 3′UTR, encoding a predicted protein of 534 amino acids. The conserved region in *jsbStAR*: N-terminal mitochondrial targeted residues, conserved phosphorylation motifs for protein kinase A [9,44] and putative amino acid residues directly contributing to the hydrophobic tunnel structure [45] were showed in Supplementary Fig. 1A. In addition, the conserved region in *jsbAMH*: the *AMH* domain and the TGF-beta domain at the C-terminus were also labeled in Supplementary Fig. 2A. Comparison of the deduced amino acid sequences of these two genes with other available sequences in NCBI evidenced a high degree of conservation among teleosts (Supplementary Figs. 1B and 2B). Phylogenetic analyses of vertebrate *StAR* and *AMH* proteins (Supplementary Figs. 1C and 2C) clearly showed that the Japanese sea bass sequences grouped with those of other teleosts, supporting the notion that they are homologous to those of other piscine species.

The resulting full-length sequences were submitted in GenBank with accession numbers KC534882 for *jsbFTZ-F1a*, KC990909 for *jsbFTZ-F1b*, JQ995529.1 for *jsbStAR* and JQ290346.1 for *jsbAMH*.

3.4. mRNA expression of jsbStAR, jsbAMH and jsbFTZ-F1 gene in various tissues of male Japanese sea bass

Tissue distributions of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* mRNA were examined using semi-quantitative RT-PCR method (Fig. 5). The *jsbAMH* and *jsbFTZ-F1* were expressed in all tissues tested, while *jsbStAR* showed a more restricted pattern of expression.

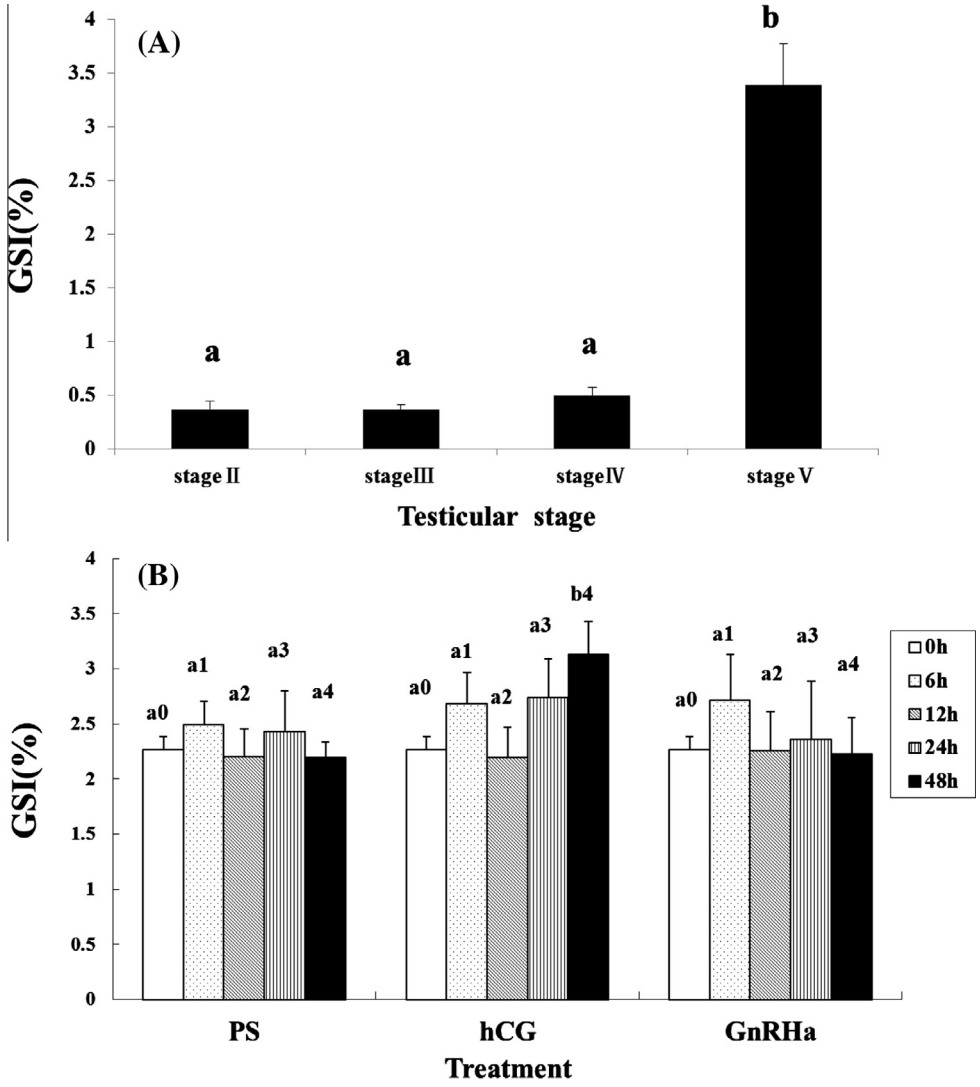


Fig. 2. The changes of GSIs in testicular development cycle (A, $N = 4$ for stage II, $N = 4$ for stage III, $N = 5$ for stage IV and $N = 11$ for stage V) and hormone administrations (B, $N = 4$) in male Japanese sea bass. Values of GSIs are expressed as mean ± standard error. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple tests).

Briefly, *jsbAMH* transcripts present highly in brain, testis, head kidney and liver and *jsbFTZ-F1* transcripts highly expressed in brain, pituitary, testis, liver and muscle. However, strong *jsbStAR* signals were obtained in head kidney and testis, and weaker signals were found in pituitary and brain. In addition, the levels of *18S* rRNA were used as internal control and were found in all tissues studied at a similar intensity.

3.5. The changes of jsbStAR, jsbAMH and jsbFTZ-F1 mRNA profiles during testicular development cycle

Transcript levels of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* showed large changes during the course of testicular development (Fig. 6). *jsbStAR* mRNA levels were strongly increased during stages III–V, peaking at stage V with levels of 4.5-fold higher than those of stage II ($P < 0.01$). The transcript levels of *jsbFTZ-F1* demonstrated a similar trend with *jsbStAR*, except that *jsbFTZ-F1* peaked at stage IV with 3.5-fold higher than stage II ($P < 0.05$). On the contrary, a progressive decline in *jsbAMH* mRNA levels was observed during spermiogenesis, particularly at stage V, when they descended to 0.7-fold of the levels of stage II ($P < 0.01$).

3.6. Regulation of the expressions of jsbStAR, jsbAMH and jsbFTZ-F1 in testis by hCG and GnRHa administration

Changes in the relative abundances of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* in response to hCG and GnRHa were assessed using quantitative real-time PCR analysis (Fig. 7). It showed that levels of *jsbStAR* in hCG and GnRHa treatments at 12 and 24 h were between 2- and 2.5-fold over the PS-injected controls, Interestingly, *jsbStAR* was strongly stimulated in response to these two hormones at 48 h, showing approximately 5.3-fold and 4.2-fold increase over controls, respectively (Fig. 7A). In the case of *jsbAMH*, significant decreases were detected in the hCG group at 24 h and GnRHa group at 48 h, what's more, *jsbAMH* relative transcript level was extremely low when compared to the control and GnRHa-treatment group at 48 h (0.17-fold and 0.32-fold, respectively, $P < 0.01$) (Fig. 7B). The *jsbFTZ-F1* mRNA level progressively, but not statistically increased in hCG-treatment group at 12 h (Fig. 7C). However, the significance can be detected in GnRHa-treatment group at the same time ($P < 0.05$). In addition, the genes (*jsbStAR*, *jsbAMH* and *jsbFTZ-F1*) transcript levels remained relatively constant during the whole administration in PS-treatment groups.

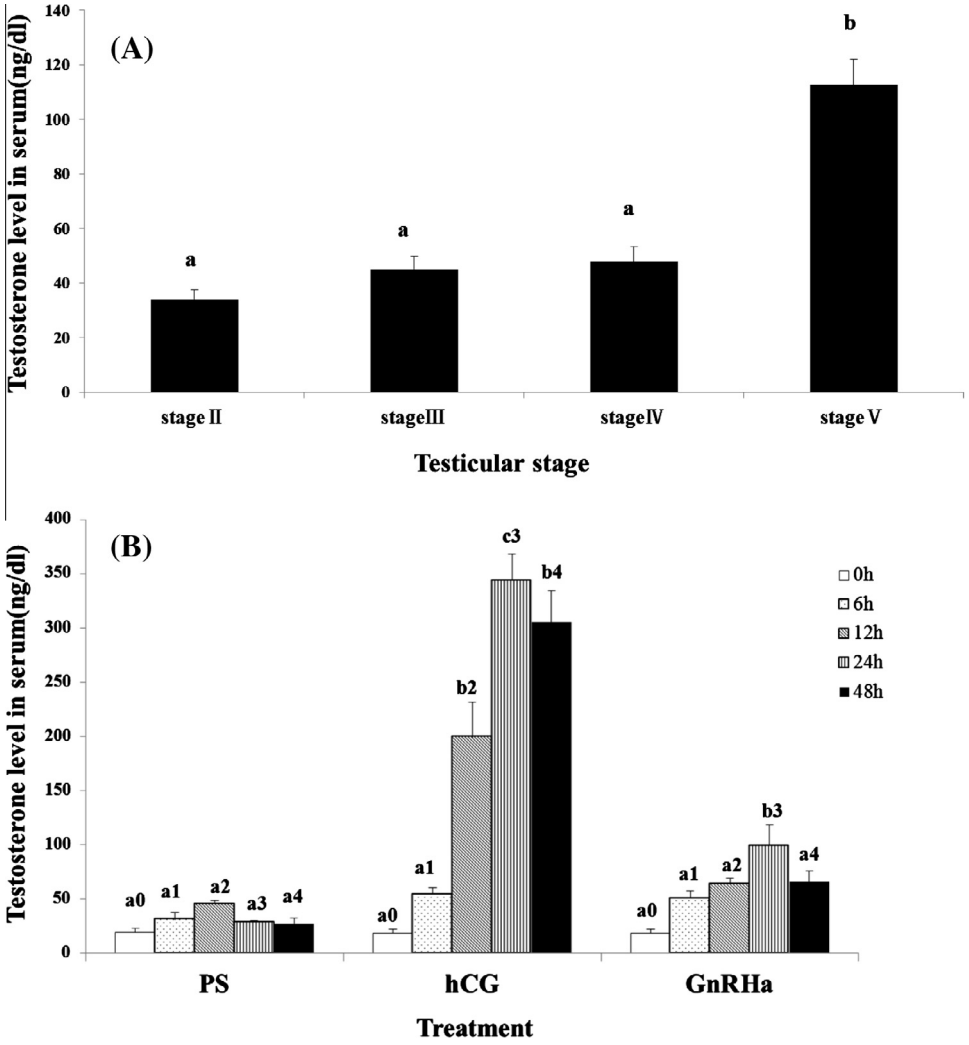


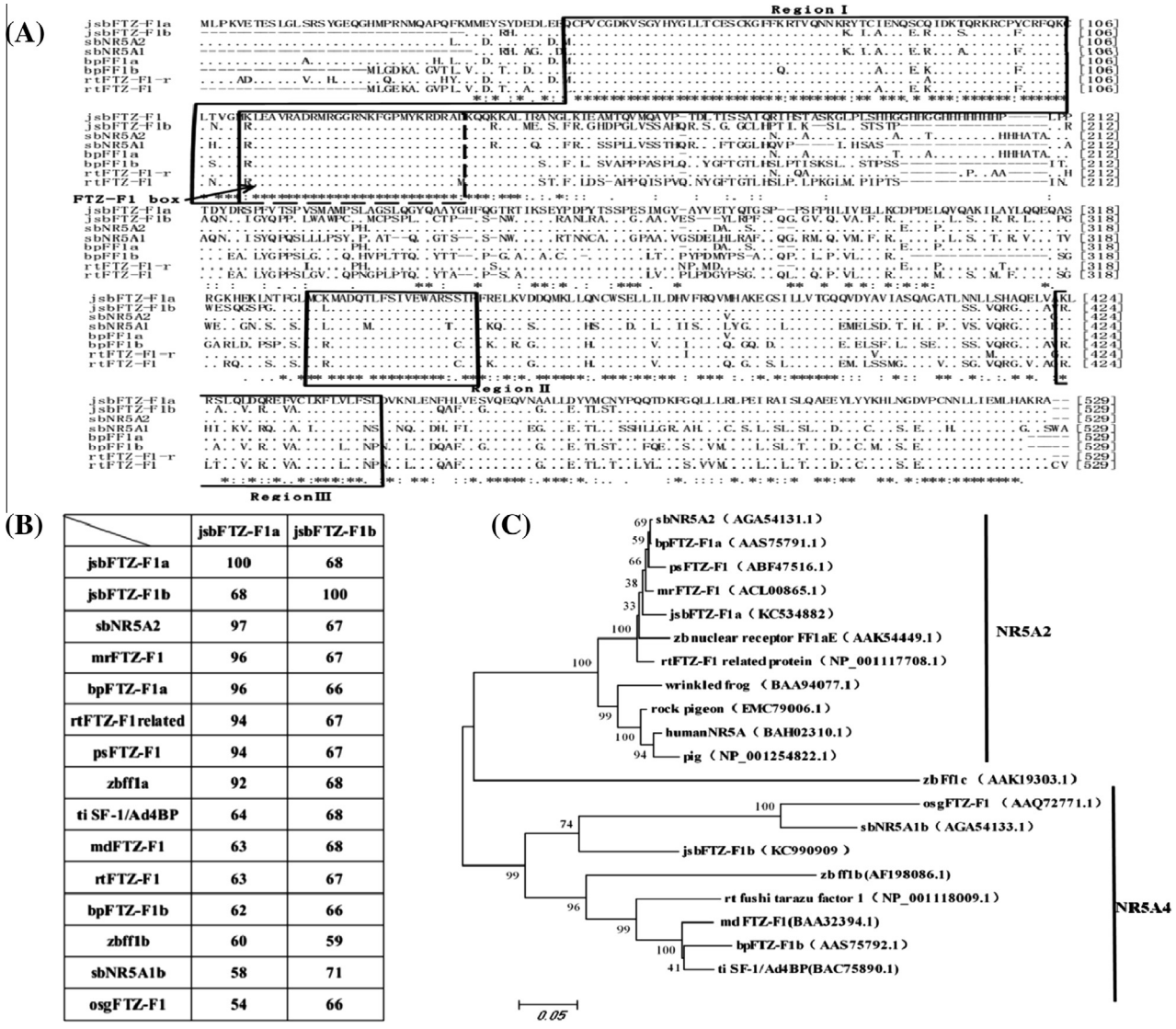
Fig. 3. The changes of testosterone levels during testicular development cycle and hormone administrations in male Japanese sea bass. (A) Changes of testosterone levels during testicular development cycle. $N = 4$ for stage II, $N = 4$ for stage III, $N = 5$ for stage IV and $N = 11$ for stage V. (B) Effects of hCG and GnRHa administration on serum testosterone levels. All values are means ± SEM ($N = 4$) and values with dissimilar letters indicate significant differences between each group on same time after PS/hormones injection ($P < 0.05$, Duncan's multiple tests).

4. Discussion

In the present study, we reported the molecular cloning and expression patterns of *StAR*, *AMH* and *FTZ-F1* from Japanese sea bass testes during testicular development. Furthermore, we studied the hCG and GnRHa-induced changes of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* genes expression in late-spermatogenesis testes and searched for relationships between their expression profiles, GSI and serum profile of testosterone.

Moving cholesterol from the outer to inner mitochondrial membrane is the critical rate limiting point in the steroidogenic pathway and is regulated by the steroidogenic acute regulatory protein (*StAR*) [4,6]. It can augment the synthesis of steroid product 5- to 10-fold [46]. Although it is well established that *StAR* gene expression is a ubiquitous characteristic of gonadal and adrenocortical tissue [8,47,48], weak signals can also be detected in brain and pituitary tissues in Japanese sea bass (Fig. 5), *StAR* mRNA has been detected in nervous tissue of other fish species, i.e., zebrafish head [47] and brains of the Japanese eel [49] and freshwater stingrays (*Potamotrygon motoro*) [50]. Further studies will be required to verify whether the *StAR* in Japanese sea bass brain has the capacity to produce neurosteroids de novo like other fish [51] and whether the *jsbStAR* transcript in brain changes during testicular development cycle and hCG/GnRHa administration. In Japanese sea bass, the quantification of *StAR* transcripts during testicular development cycle (Fig. 6) revealed a trend similar to their GSIs (Fig. 2A) and serum testosterone levels (Fig. 3A), remaining relatively constant during early spermatogenesis then markedly increased as spermatids transformed into spermatozoa. These results were in line with those reported in European sea bass [52]. In addition, the significant increase of *jsbStAR* expression at 12–48 h in hCG and GnRHa-treatment groups (Fig. 7A) also illustrated similar trends with their GSIs (Fig. 2B) and their testosterone levels (Fig. 3B). Our results suggest that *jsbStAR* may contribute to the hCG/GnRH-stimulated androgen production in the Japanese sea bass testis.

In vertebrates, a multitude of studies have shown that anti-Müllerian hormone exerted inhibitory effects on male and female gonadal steroidogenesis and differentiation [20], and the presence of *AMH* in testis, concretely in the Sertoli cells has also been well-described in mammals [53,54], birds [55] and reptiles [56,57]. The first piscine *AMH* was found in eel, termed 'eel spermatogenesis related substances 21' (eSRS21), which was down-regulated by hCG and mainly expressed in Sertoli cells of immature testes [10]. In the present study, *jsbAMH* mRNA was also highly expressed in testis (Fig. 5) and during the testicular development cycle, high



	jsbFTZ-F1a	jsbFTZ-F1b
jsbFTZ-F1a	100	68
jsbFTZ-F1b	68	100
sbNR5A2	97	67
mrFTZ-F1	96	67
bpFTZ-F1a	96	66
rtFTZ-F1related	94	67
psFTZ-F1	94	67
zbff1a	92	68
ti SF-1/Ad4BP	64	68
mdFTZ-F1	63	68
rtFTZ-F1	63	67
bpFTZ-F1b	62	66
zbff1b	60	59
sbNR5A1b	58	71
osgFTZ-F1	54	66

Fig. 4. Comparative analysis of Japanese sea bass *FTZ-F1a* and *FTZ-F1b* genes. (A) Full amino acid sequences of sbNR5A2 (GenBank Accession No. AGA54131), sbNR5A1 (AGA54133), bpFF1a (AAS75791), bpFF1b (AAS75792), rtFTZ-F1-r (NP_001117708) and rtFTZ-F1 (NP_001118009) are aligned with CLUSTAL W software. Identical amino acids are indicated by asterisks (*), conservative substitutions are shown by colons (:) and semiconservative substitutions are indicated by commas (.). Regions I, II, III (solid line) and the *FTZ-F1* box (dotted line) are indicated in boxes. (B) Percent homology of the deduced amino acid sequences of the cloned Japanese sea bass *FTZ-F1a* and *FTZ-F1b* with those from other species. The Genbank accession numbers are showed in (C). (C) Phylogenetic analyses are conducted in MEGA5.0. Protein sequences used for comparison and their Genbank accession numbers are listed at the right of the branches. Complete names of vertebrate used in Fig. 4 are displayed as follows: sb: European sea bass (*Dicentrarchus labrax*); mr: mangrove rivulus (*Kryptolebias marmoratus*); bp: black porgy (*Acanthopagrus schlegelii*); rt: rainbow trout (*Oncorhynchus mykiss*); ps: patagonian silverside (*Odontesthes hatcheri*); zb: zebrafish (*Danio rerio*); ti: tilapia (*Oreochromis niloticus*); md: medaka (*Oryzias latipes*); osg: orange-spotted grouper (*Epinephelus coioides*); winkled frog: *Glandirana rugosa*; rock pigeon: *Columba livia*; pig: *Sus scrofa*; human: *Homo sapiens*.

level of *jsbAMH* mRNA expressed in immature testes until spermatogonia started to proliferate intensively and decreased in maturing testes (Fig. 6). The similar expression pattern has also been found in zebrafish, Japanese flounder and European sea bass [12,13,22,58]. This is the first time that a down-regulation of *AMH* gene expression was observed in maturing male Japanese sea bass. In addition, the *jsbAMH* mRNA expressions displayed a striking different profile when comparing to serum concentrations of testosterone and the reciprocal relationship between them can be detected both in testicular development cycle and hCG/GnRHa administration (Figs. 6 and 7B). This relationship has also been found in some male species [19]. It has been confirmed that testosterone is produced in the Leydig cells of the testis upon stimulation by LH, activating adenylyl cyclase and the subsequent cAMP-stimulated signal transduction pathway [59]. Otherwise, *AMH* inhibited the expression of steroidogenic enzyme, i.e., aromatase [12,60,61], P450 C17α-hydroxylase/C17–20 lyase [62] and negatively modulated the differentiation and function of Leydig cells [63]. These results suggest that *jsbAMH* may involve in inhibiting testosterone production in Japanese sea bass Leydig cell during testicular development cycle and hCG/GnRHa administration. A range of transcription elements in *AMH* promoter sequence, i.e., GATA [64], SOX9 [20,65], SF-1 [33,13] and WT-1 [66,67] have been proven their functionality in *AMH* by in vitro and in vivo studies, while the SF-1 is considered to be the key regulator of *AMH* transcription [13,68]. Our further efforts are to definitely understand the molecular mechanisms regulating *AMH* expression and its signaling in piscine Leydig cell.

In several teleost, such as rainbow trout, black porgy, European sea bass and zebrafish, two or more separate *FTZ-F1* homologues have been cloned [22,27,30,32,69,70], of which one group can be placed in NR5A2 clade, but the other group is mainly aligned

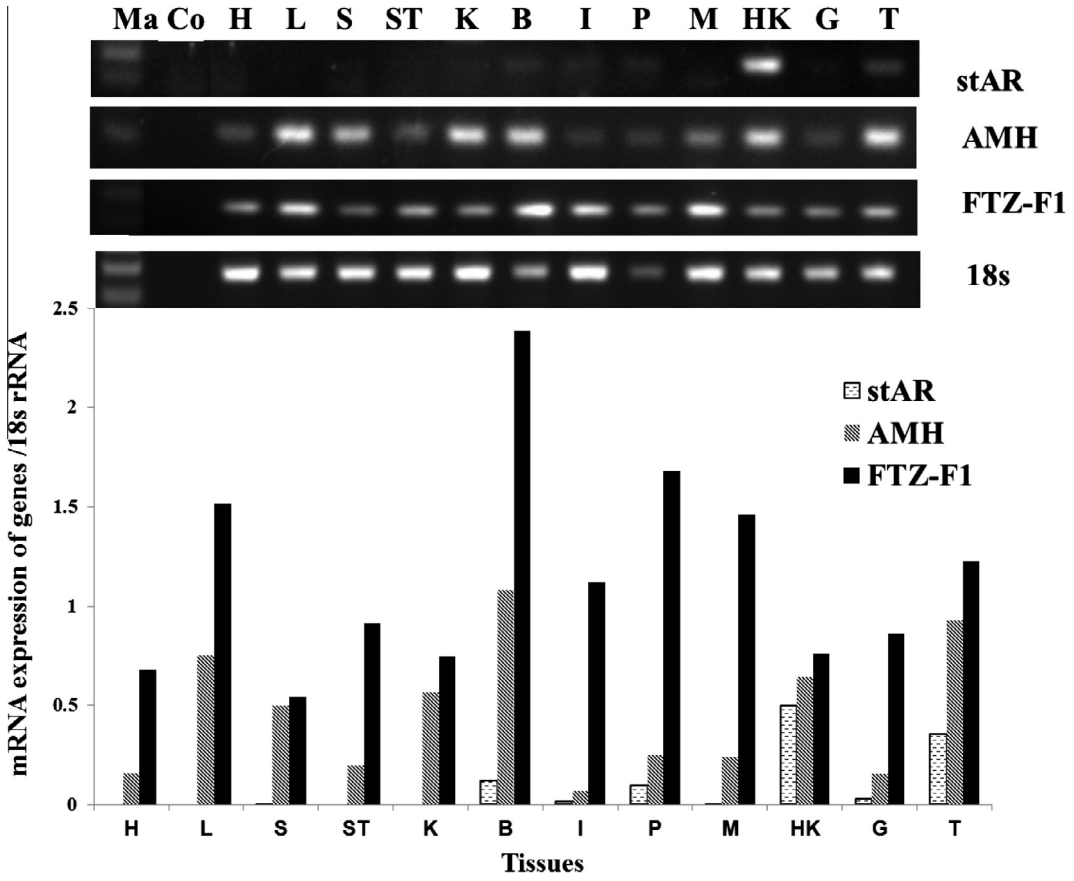


Fig. 5. Tissue expression patterns of the *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* in adult male Japanese sea bass. *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* genes expression profiles were analyzed by RT-PCR using specific primers and *18S* ribosomal RNA was used as internal control for relative quantity. DNA molecular weight marker (Ma), control (Co, using water as template), heart (H), liver (L), spleen (S), stomach (ST), kidney (K), brain (B),intestine (I), pituitary (P), muscle (M), head kidney (HK), gill (G) and testis (T).

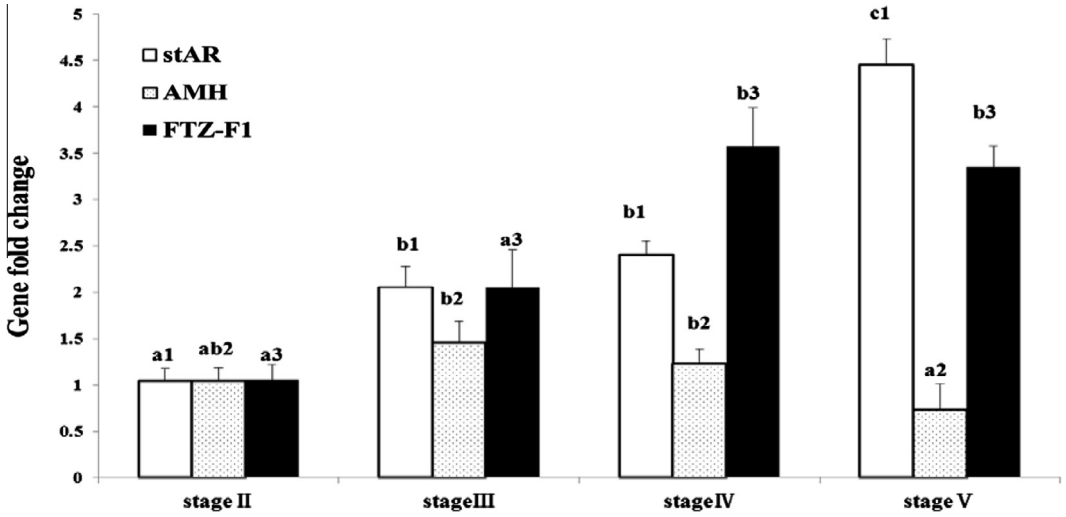


Fig. 6. mRNA expressions of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* in Japanese sea bass during testicular development cycle. *18S* ribosomal RNA was used as internal control gene. Data are expressed relative to stage II. Values are expressed as mean ± standard error of mean. Values with different letters indicate statistical significance by one-way ANOVA, Duncan's multiple tests.

within *FTZ-F1*-related proteins (NR5A4) which only has been shown in teleosts so far. It was different from the mammalian classify criteria, NR5A1 and NR5A2. This suggests that different classify criteria for teleosts is necessary [22]. In Japanese sea bass, we also found two *FTZ-F1* homologues which belong to different clades (NR5A2 and NR5A4) (Fig. 4C). What's more, although the *tAd4BP/SF-1* of tilapia, *FTZ-F1* homologue of arctic char (*Salvelinus alpinus*), *ff1a* of zebrafish and *mdFTZ-F1* of medaka belong to different clads, they were all detected to regulate the transcription of enzymes involved in steroidogenic pathways [30,33,34], according to these results, we analyzed the whole *FTZ-F1* by designing the primers in the *FTZ-F1* conservative region in present study. The

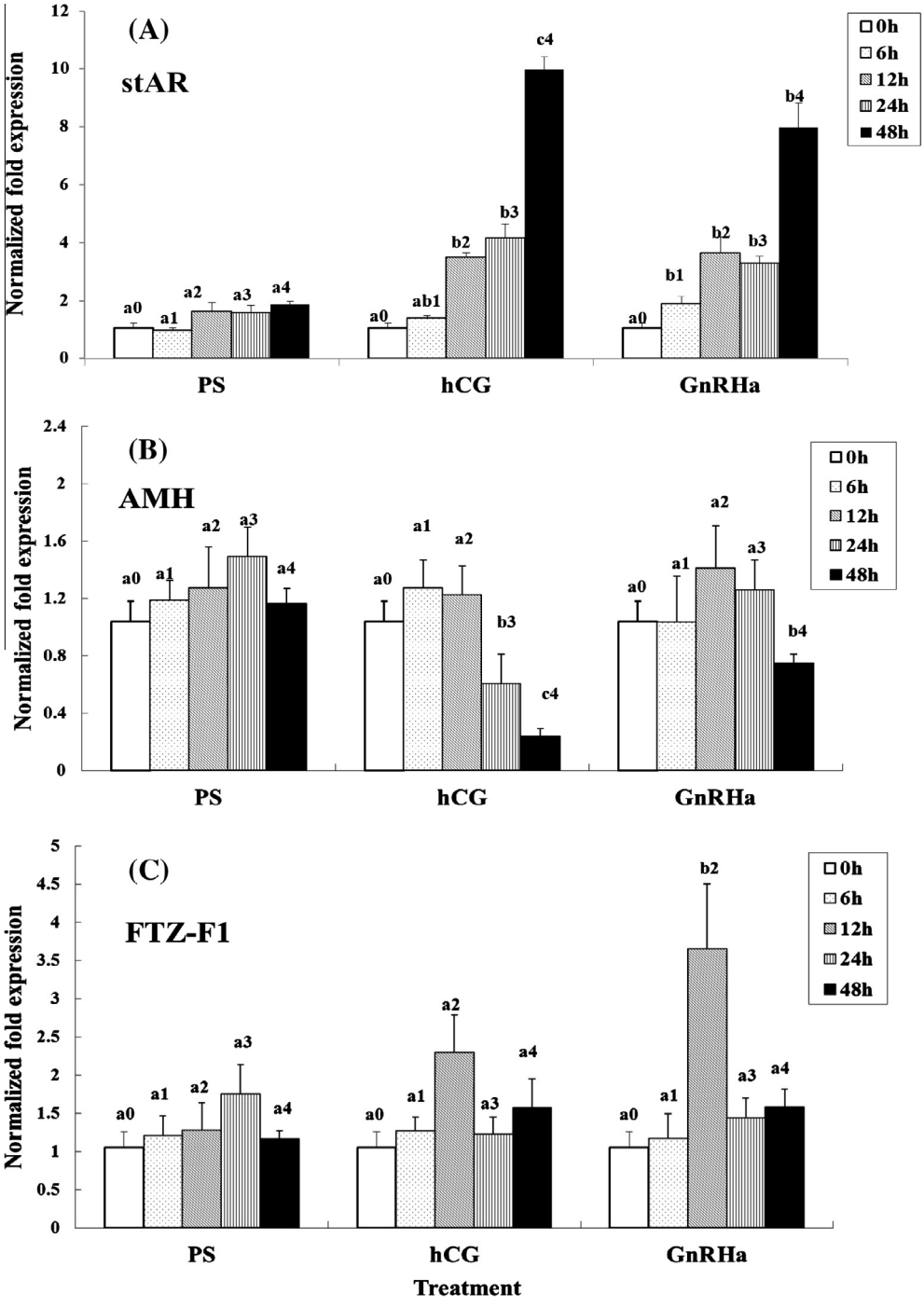


Fig. 7. Regulation of the *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* expressions in Japanese sea bass testis by hCG and GnRHa treatment in vivo. The expressions of these genes were analyzed by qPCR. Data are expressed relative to control, PS-injected fish (mean ± SE). Different letters are indicated significant differences between each group on same time after PS/hormones injection ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

jsbFTZ-F1 was expressed in a broad variety of tissues and transcripts were particularly high in brain, testis, liver and pituitary (Fig. 5). Similar to *jsbFTZ-F1*, other *FTZ-F1* genes of teleosts also expressed in tissues which involved in functions related to both steroidogenesis and cholesterol transfer and metabolism [27,29,30]. Furthermore, the current study showed that the highest *jsbFTZ-F1* mRNA profile was detected at stage IV (Fig. 6), while the highest testosterone level was found at stage V (Fig. 3A). In additon, the expression level of *jsbFTZ-F1* mRNA significantly increased at 12 h after treating with GnRHa (Fig. 7C), after 24 h, the serum testosterone was also found to increase (Fig. 3B). This activation has also been described in black porgy [37] in which the mRNA levels of *SF-1* in gonad were high at 6 h post GnRHa injection then plasma testosterone significant increased after 24 h. These results suggest that the *FTZ-F1* may involve in testosterone synthesis in Japanese sea bass by activating reproductive axis.

The orphan nuclear receptors, particularly SF-1, are known to regulate gene expression related to gonadotropins and steroidogenesis in mammal [71–74]. In zebrafish, *FTZ-F1* homologue (ff1b) has been designated a functional homolog of mammalian SF-1 [75]. In this study, the elevated *jsbFTZ-F1* mRNA levels

preceded increasing *jsbStAR* and decreasing *jsbAMH* transcriptions during testicular development cycle and hCG/GnRHa administration (Figs. 6 and 7). For *StAR*, many studies have identified the potential SF-1 site in its 5′-flanking region [76]. *Ff1b* mRNA levels in zeabrafish were correlated with *StAR* mRNA levels during the initiation of spermatogenesis [75] and the link between gonadal expression of *acFF1* and *StAR* was also observed in the reproductive process of Arctic char [30]. These results suggest *FTZ-F1* may involve in the transactivation of the *StAR* [76]. With regard to *AMH*, *FTZ-F1* was wildly supposed to be crucial in mammalian steroidogenesis by regulating *AMH* expression [20,21]. The temporal correlation between SF-1 and MIS gene expression [77] and the potential SF-l-responsive element in its 5′-flanking region showed the probably important role of *FTZ-F1* for the initiation of *AMH* transcription [68,77,78]. Furthermore, the coexpression of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1* observing in head kidney, testis, pituitary and brain (Fig. 5) also suggest the potential role of *FTZ-F1* in steroid production pathways by regulating the expression of *StAR* and *AMH* in Japanese sea bass. In addition, in many teleosts, CYP19a gene was verified to be regulated by *FTZ-F1*, as potential *FTZ-F1*-binding sites were observed in its 5′-flanking regions [34,79–81]. A strong correlation between P450scc and Ff1b in zebrafish suggested that the transcription of P450scc may also require *FTZ-F1* [27]. Thus, *FTZ-F1* may play an important role in Japanese sea bass by regulation of steroidogenic enzyme expression.

In summary, this study described for the first time the reproductive physiology of Japanese sea bass in molecular level. It provided an overall picture of changes in steroidogenic relational genes (*StAR*, *AMH* and *FTZ-F1*) expressions during testicular development cycle and hCG/GnRHa treatment. The patterns of testosterone secretion appeared to be largely regulated by changes in expression of *jsbStAR*, *jsbAMH* and *jsbFTZ-F1*. In addition, our results highlighted the importance of hCG and GnRH, especially the hCG, in regulating the progression of spermatogenesis in the Japanese sea bass. Further in vitro and in vivo studies will be needed to understand how gonadotropins, sex steroids and other gonadal factors (P450C17 or 3β-HSD) interact to regulate Japanese sea bass reproduction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.03.014.

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