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Cloning, characterization, and expression analysis of a putative 17 beta-hydroxysteroid dehydrogenase 11 in the abalone, *Haliotis diversicolor supertexta*

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ABSTRACT

The 17-beta-hydroxysteroid dehydrogenases (17β-HSDs) are key enzymes for sex steroid biosynthesis. To date, relatively little is known about the presence and function of 17β-HSDs in marine gastropods. In the present study, a cDNA sequence encoding putative 17β-HSD type 11 (17β-HSD-11) was identified in marine abalone (*Haliotis diversicolor supertexta*). The full-length cDNA contains 1058 bp, including an open reading frame (ORF) of 900 bp that encodes a protein of 299 amino acids. Comparative structural analysis revealed that abalone 17β-HSD-11 shares relatively high homology with other 17b-HSD-11 hormologues, and a lesser degree of amino acid identity with other forms of 17b-HSD, especially in the functional domains, including the cofactor binding domain (TGxxxGxG) and catalytic site (YxxSK). Phylogenetic analysis showed that abalone 17β-HSD-11 belongs to the short-chain dehydrogenase/reductase (SDR) family. Functional analysis following transient transfection of the ORF into human embryonic kidney-293 (HEK-293) cells indicated that abalone 17β-HSD-11 has the ability to convert 5α-androstane-3α,17β-diol (3α-diol) to androsterone (A) and testosterone (T) to androstenedione (4A). Expression analysis *in vivo* demonstrated that abalone 17β-HSD-11 is differentially expressed during three stages (non-reproductive, reproductive, and post-reproductive). Taken together, these results indicate that ab-17β-HSD-11 is an SDR family member with a potential role in steroid regulation during the reproductive stage.

1. Introduction

17-beta-hydroxysteroid dehydrogenases (17β-HSDs) are remarkably multifunctional enzymes that mediate the metabolism of sex steroids. The enzymes catalyze oxidation and reduction at position C17 of C18- and C19-steroids in which hydroxyl groups are transformed into keto groups and *vice versa.* In these steroids hydroxy-forms are more active, whereas keto-forms are relatively inactive [1–4]. Almost all 17β-HSDs belong to the superfamily of short-chain dehydrogenases/reductases (SDRs) except 17β-HSD type 5, which is a member of the aldoketo-reductase (AKR) protein family. At present, 15 types of 17β-HSDs have been identified in vertebrates, and they have been numbered according to the order of their discovery [5]. Typical 17β-HSDs share four relatively conserved motifs, consisting of the cofactor binding domain TGxxxGxG, the catalytically active sites YxxSK, the structural stabilization domain NAG, and the PGxxxT domain that determines the reaction direction [6–8]. Substrate preferences of 17β-HSDs are various and not restricted to steroids, so different types of 17β-HSDs might possess various physiological functions in the steroid metabolism-related signaling pathways [9,10].

It is well known that 17β-HSDs play a pivotal role in the steroidogenic pathways in mammalian species. In recent years, 17β-HSDs have also been found to participate in biological functions that are important for steroid metabolism in aquatic fish, such as the zebrafish and Japanese eel [11,12]. Increasing numbers (or isoforms) of the 17β-HSD family are being isolated and identified because of their diversity [13], but, to the best of our knowledge, relatively little information is known about 17β-HSDs in mollusks. Porte et al. reviewed the presence of 17β-HSD activity in mollusks, including bivalves, cephalopods, and gastropods [14]. Given the lack of knowledge of mollusk endocrinology, Porte et al. deemed it

Abbreviations: 17β-HSD-11, 17β-hydroxysteroid dehydrogenase 11; SDRs, short-chain dehydrogenases/reductases; E1, estrone; E2, estradiol; 3α-diol, 5α-androstane-3α,17β-diol; A, androsterone; T, testosterone; 4A, androstenedione; DMSO, dimethyl sulfoxide; ORF, open reading frame; HEK-293, human embryonic kidney 293 cells; qPCR, real-time quantitative PCR.

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is necessary to study endocrine-related genes, such as 17β-HSDs. They summarized some of the steroid-related genes in mollusks, such as 17β-HSD types 3, 5, and 9. However, the other members of the 17β-HSD family (for example, type 11) have not been functionally characterized and their genes have not been cloned. In our previous work, we demonstrated that 17β-HSD type 12 participates in steroid-mediated physiological processes in abalones (a marine mollusk) [15]. We now focus on 17β-HSD type 11 (17β-HSD-11) in this work, to assess its molecular characteristics and biological function in mollusks.

17β-HSD-11 was initially discovered by Li et al. when searching for a novel enzyme in glucocorticoid metabolism, as it is homologous to other 17β-HSDs and has the ability to transform 17β-hydroxysteroid hormones [16]. Chai et al. found that the enzyme is similar to human retinal short-chain dehydrogenase/reductase (retSDR2) but cannot catalyze the metabolism of retinoids, although the enzyme can bind to them [17]. 17β-HSD-11 can catalyze the conversion from 5α-androstane-3α,17β-diol (3α-diol) to androsterone (A), and transforms testosterone (T) into androstenedione (4A) as well [17,18]. Recent investigations demonstrated that 17β-HSD-11 can be up-regulated significantly by peroxisome proliferator-activated receptor (PPARα) agonists in mouse intestine, indicating that this enzyme is also involved in the lipid metabolic pathways of mouse [19–21]. 17β-HSD-11 has a broad tissue distribution in humans as it was detected in the pancreas, kidney, liver, and lung as well as steroidogenic cells [17], and is also found in an androgen-dependent prostate cancer cell line [22,23].

Although quite a few studies concerning 17β-HSD-11 in vertebrates have been published, little investigation of 17β-HSD-11 in invertebrates, especially marine mollusks, has been launched. Whether 17β-HSD-11 exists in these species and plays the same or similar roles as its counterpart in vertebrates remains unclear. In this study, we screened a putative cDNA of 17β-HSD-11 from an abalone digestive gland cDNA library by data mining the EST database. The ability of the enzyme to catalyze the transformation of steroid substrates was then assessed by transiently transfecting the candidate gene (*i.e.*, 17β-HSD-11) into HEK-293 cells. The aims of this study were to identify the sequence and characterize the catalytic activities of ab-17β-HSD-11 and, consequently, provide evidence for further investigation of the biological functions of the target gene in the steroidogenic pathway in abalone.

2. Materials and methods

2.1. Construction of the abalone digestive gland cDNA library

The full-length cDNA library from abalone (*Haliotis diversicolor supertexta*) digestive gland was constructed and 6000 EST sequences were obtained by random sequencing using universal primers described in a previous study [24]. A 369-base pair (bp) fragment that shared high sequence similarity with the 17β-HSD-11 gene of other animals was screened through a BLAST search using 17β-HSD-11 genes from other animals, and this sequence was selected for further study.

2.2. Cloning the full-length cDNA of 17β-HSD11

The candidate sequence of 17β-HSD-11 was analyzed by ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the results indicated that the sequence contains the 5′ end coding region, which has a methionine at the start of the ORF preceded by a short UTR. A forward primer F1 (5′-CCA CCC GCT AGA CAA GAT-3′) was then designed to obtain the 3′ end of 17β-HSD-11 using anchored PCR with the universal reverse primer M13R. PCR amplification was performed in a 20-μL reaction volume containing 2.0 μL of 10× PCR buffer, 1.0 μL of $MgCl_2$ (25 mM), 1.0 μL of dNTPs (2.5 mM), 1.0 μL of each primer (10 mM), 0.1 μL of ExTaq polymerase (5 U mL^{-1}) (Takara, Dalian, China), and 1 μL of cDNA mixture, with DEPC-treated water making up the volume to 20 μL. The touch-down PCR procedure was performed as follows: 1 cycle of 94 °C for 5 min, then 10 cycles of 94 °C for 30 s, 67 °C for 30 s (−0.7 °C per cycle), 72 °C for 1 min, then 26 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by 1 cycle of 72 °C for 6 min. The PCR product was purified and subcloned into pMD18-T vector (Takara, Dalian, China), and then transformed into the *E. coli* M15 competent cells. The plasmid of positive clones was purified and sequenced by an ABI3730 Automated Sequencer (ABI, USA) using primer F2 (5′-CTG TGA CGT CAC TTC CAC CGA-3′) and M13R primer. By compiling the overlapping sequence of the PCR product and the known sequence, the complete 17β-HSD-11 sequence was obtained. Subsequently, another PCR was carried out to verify the 17β-HSD-11 sequence using a new cDNA template prepared from the primers F3 (5′-ATA TGA AAG CTT CTA TTG GA-3′) and R4 (5′-GTA TCA GTC ACT CTT TGC GT-3′) located in the 5′- and 3′-UTR, respectively. This verified sequence was denoted as ab-17β-HSD-11. All primers applied in this work were synthesized by Invitrogen Corporation, Shanghai Representative Office.

2.3. Sequence analysis

The sequence similarities between ab-17β-HSD-11 and other known genes were analyzed using the BLAST program (http://www.ncbi.nim.nih.gov/blast). The Expert Protein Analysis System (http://www.expasy.org/) was used to analyze the protein sequence, including determination of the primary structure and prediction of the second structure and subcellular localization. Protein multiple alignments were performed using ClustalX 1.83 software [25]. Phylogenetic trees were constructed using MEGA4.1 software with 1000 bootstrap trials by the neighbor-joining method [26].

2.4. Construction of ab-17β-HSD-11 expression vector in pFLAG-cmv2

The ab-17β-HSD-11 expression vector was constructed in pFLAG-cmv2. Briefly, specific primers F5 (5′-ATG CGG CCG CTA TGA AAG CTT CTA TTG GA-3′) and R6 (5′-GAC CCG GGT CAG TCA CTC TTT GCG T-3′), containing a NotI and a SmaI restriction site (underlined), respectively, were used to amplify ab-17β-HSD-11. PCR was performed for 30 cycles, with denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C, and elongation for 90 s at 72 °C. The PCR product was gel-purified, digested with NotI and SmaI restriction endonucleases, and then subcloned into the pFLAG-CMV2 vector. Then, the constructed plasmid was transformed into *E. coli* DH5α competent cells. The positive clones were screened by PCR using primers F5 and R6. The integrity of the construct, pFLAG-cmv2-ab-17β-HSD-11, was verified by sequencing the inserted DNA fragment using pFLAG-cmv2 universal primers F7 (5′-TTC CAA AAT GTC GTA ATA AC-3′) and R8 (5′-ATT ATA GAA GGA CAC CTA GTC-3′).

2.5. Transient transfection of pFLAG-cmv2-ab-17β-HSD-11

The plasmid pFLAG-cmv2-ab-17β-HSD-11 was purified using the Plasmid DNA Maxi Purification Kit (free-endotoxin) (OMEGA, Bio-Tek, USA). HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) containing 10% (v/v) fetal bovine serum (FBS) (Invitrogen, USA) and penicillin/streptomycin at 37 °C with 5% CO_2. Cells were seeded into 24-well culture plates (0.8×10^5/well) 36 h prior to transfection and transiently transfected with the construct pFLAG-cmv2-ab-17β-HSD-11 or empty

vector using Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA), according to the manufacturers' protocol (0.8 μg DNA and 2.0 μL Lipofectamine™ 2000 were transfected per well). Twenty-four hours after transfection, six substrates (3a-diol, A, T, 4A, estrone (E1), or estradiol (E2)) at concentrations of 5 μg/mL were added into ab-17β-HSD-11-transfected cells, respectively. These steroid substrates were firstly dissolved in DMSO, then the desired concentration (5 μg/mL) was obtained by serial dilution. The culture media from each well were collected after 23 h and were analyzed for precursor and product steroids by TLC or HPLC. At the same time, mock-transfected cells were used as controls in the same assay. Each experiment was performed with three replicates.

2.6. Detection of steroid substrates by TLC and HPLC

Steroid metabolites were extracted from cull culture media and analyzed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Briefly, the supernatant from each treatment was centrifuged at 13,800 × *g* for 10 min at 4 °C to precipitate the residual cells and other impurities. Then each of the supernatant fluids was extracted using two volumes of a mixture (9:1, v/v) of methyl-tert-butyl ether and methanol. After vortexing thoroughly (1 min) and allowing to stand (5 min), the upper organic phase was carefully transferred into another clean Eppendorf tube. Subsequently, the extractant was evaporated by nitrogen flow at ambient temperature prior to further analysis.

TLC analysis was performed according to a previously published procedure [27], with some modifications. Briefly, each sample was dissolved in 50 μL chloroform/methanol (2:1, v/v), and 10-μL aliquots were spotted on 10 × 20 cm high silica gel GF pre-coated TLC plates (Jiangyou Gel Co., Ltd., Yantai, China) together with 0.5 μg of each of the carrier steroids (3a-diol and A). The plates were developed in chloroform/toluene/acetone (50:80:20, v/v/v) for 1 h and air-dried. Steroids were visualized by spraying with 5% sulfuric acid in ethanol and heating the plate at 105 °C for 9 min.

For separation by HPLC, each sample was dissolved in a 250-μL mixture of acetonitrile–water (7:3, v/v) and 10 μL were injected into a LC-2010 HPLC system equipped with a double-pump unit (LC-20AT), a controller (CBM-20A), an autosampler (SIL-20A), a UV detector (SPD-20A), and a 5-μm C18 Shim-pack VP-ODS column (4.6 mm × 150 mm) (Shimadzu, Japan). The samples were separated at 25 °C and a flow rate of 1 mL/min with a program that involved linear gradients of 65–70% acetonitrile in water over 2 min, 70–80% acetonitrile over 3 min, followed by 80–70% acetonitrile over 2 min. The UV detection wavelength was set at 230 nm. The retention times in minutes were as follows: E2 2.52, E1 2.80, T 2.84, and 4A 3.11. Steroids were identified by comparison with the standards. The data were measured in triplicate and activities were calculated as the percentage of substrate converted into product.

2.7. Expression analysis of ab-17β-HSD-11 in the reproductive stage

Healthy adult abalones with an average shell length of 56.0 ± 3.0 mm were procured from a local hatchery (Longgang District, Shenzhen City, China) and used for the experiments. Their tissue samples (digestive glands and gonads) were collected from three different stages (non-reproductive, reproductive, and post-reproductive). The reproductive stage is defined as when the abalone is in the process of spawning, and the post-reproductive stage refers to the seventh day after spawning; the non-reproductive stage refers to the period when adult male and female abalone were collected around 3 weeks prior to spawning, and was used as a control. Tissue samples from three males or females in each stage were pooled together and homogenized in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, USA), mRNA was purified by mRNA purification kit (BBI, Canada), and then cDNA was synthesized by MMLV (Takara, Dalian, China).

qPCR assays were performed to determine the expression of ab-17β-HSD-11 in the different reproductive stages using standard protocols on a 7300 RT-PCR System (Applied Biosystems, USA) with a 96-well reaction plate. Briefly, each 25-μL DNA amplification reaction contained 12.5 μL 2× SYBR Green Premix Ex Taq with ROX dye I (Takara, Dalian, China), 1.0 μL diluted cDNA, and 0.5 μL 10 μM solutions of each forward and reverse primer, with PCR-grade water to make up the volume. Primers (rt-F: 5′-CCA CCC GCT AGA CAA GAT-3′ and rt-R: 5′-CCA ATA CGA CGG ACC TCA-3′) for amplification of the 170-bp gene-specific product were designed according to the target gene sequence. The RT-PCR assay was done under the following conditions: 30 s at 95 °C; then 5 s at 95 °C, 31 s at 60 °C for 40 cycles; 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C as the final dissociation stage. Beta-actin, with a pair of primers ab-actin-F (5′-GTC TTT CCC TCC ATC GTC GGA C-3′) and ab-actin-R (5′-GTC CCA GTT GGT GAC GAT TCC G-3′), from *H. diversicolor supertexta* was used as an internal control to calibrate the cDNA template [15]. In addition, the expression of ab-17β-HSD-11 in the non-reproductive stage was chosen as the calibrator for normalizing different samples. Data obtained from triplicate runs for target cDNA amplification were averaged.

2.8. Statistical analysis

The HPLC data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons using SPSS 16.0. The RT-PCR data analysis was executed using SDS 2.0 software (ABI, USA) according to the $2^{-\Delta\Delta Ct}$ method [28]. All values are presented as mean ± SEM, and $P < 0.01$ was considered statistically significant.

3. Results

3.1. cDNA cloning and sequence analysis of ab-17β-HSD 11 gene

By aligning the known gene fragment and the amplified fragment of 689 bp, we acquired a 1058-bp sequence, which represented the full sequence of the putative abalone 17β-HSD-11. The sequence was named ab-17β-HSD-11 and deposited at GenBank with the accession number ADV02385. Ab-17β-HSD-11 contained an open reading frame of 900 bp encoding 299 amino acid residues, a short 5′-UTR of 31 bp, and a 158-bp 3′-UTR with a canonical polyadenylation signal sequence, AATAAA, located 9 bp upstream of the poly A tail. The molecular weight of the deduced protein is 33.45 kDa with an estimated pI of 6.97. The deduced peptide ab-17β-HSD-11 includes a catalytic domain and a variable C-terminal region. The catalytic domain contains a cofactor binding site motif (TGAGHGIG) (43–50), a putative catalytic site (YSASK) (186–190), and a typical SDR conserved domain (NNVG) (120–123).

BLAST analysis of type 11 members revealed 44%, 43%, 44%, 43%, 47%, and 53% identity with *Home sapiens* (NP_057329), *Pongo abelii* (NP_001127691), *Bos Taurus* (NP_001039751), *Mus musculus* (NP_444492), *Xenopus (Silurana) tropicalis* (NP_001011304), and *Saccoglossus kowalevskii* (XP_002732320) respectively.

3.2. Comparison of the peptide sequence of ab-17β-HSD-11 with those of various other species

BLAST analysis revealed that the deduced peptide sequence of ab-17β-HSD-11 shares a high degree of sequence similarity (39–50%) and identity (21–35%) with other 17β-HSD family members, and especially with other 17β-HSD-11s. The highest similarity (71%) and identity (53%) are shared with *S. kowalevskii* (an acorn

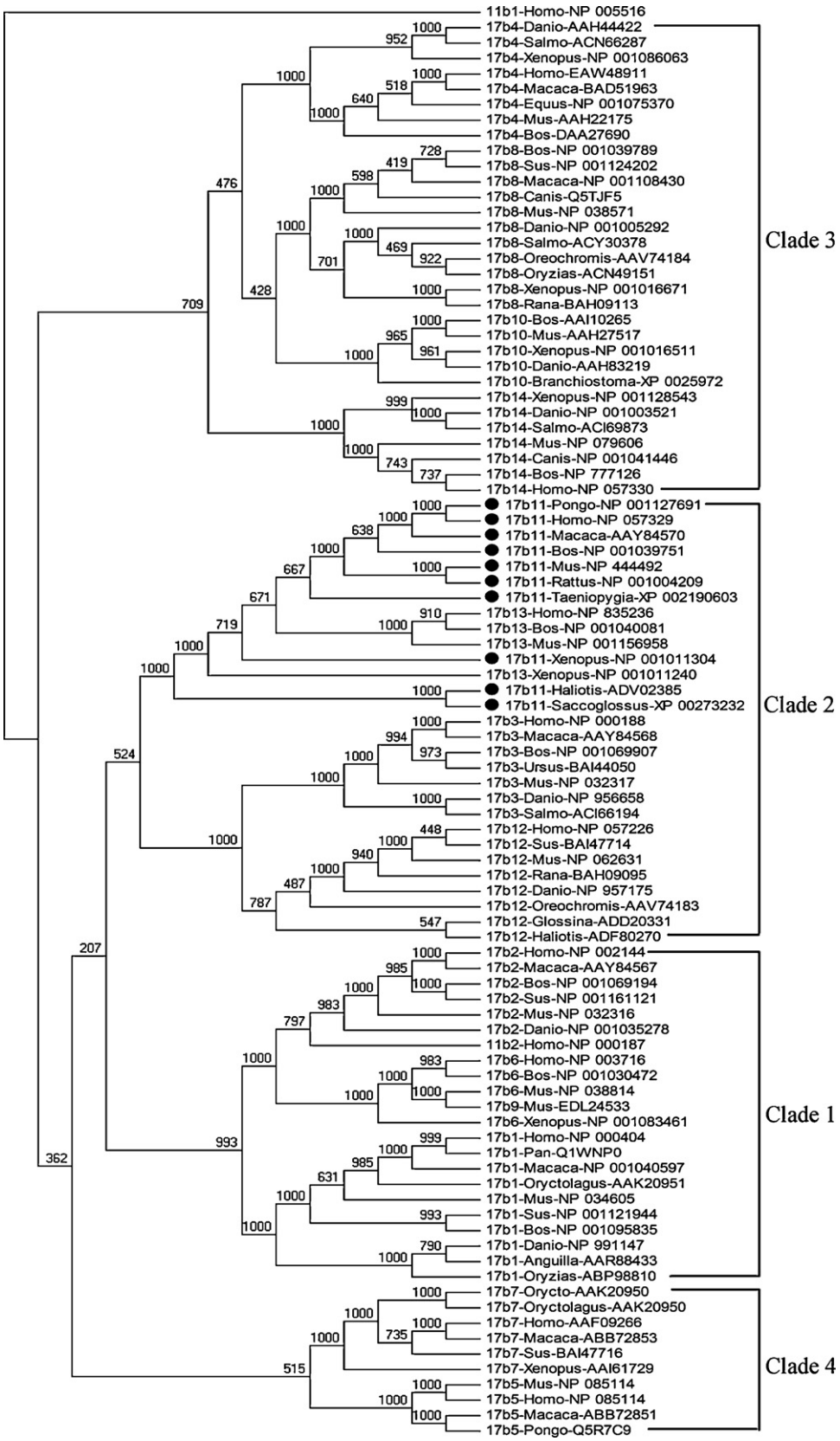

Fig. 1. Phylogenetic analysis of 17β-HSDs (from type 1 to type 14) of various species using MEGA4.1 and based on ClustalX sequence alignment. The phylogenetic tree revealed the evolutionary relationship among the 17β-HSDs. Bootstrap values, marked on each branch, represent the number of times out of 1000 that two organisms grouped together during bootstrap analysis. Branch length is proportional to the distance between each protein. The type 11 family is highlighted with solid spheres. Type 2 11β-HSD, which belongs to the SDR family, was selected as an outgroup.

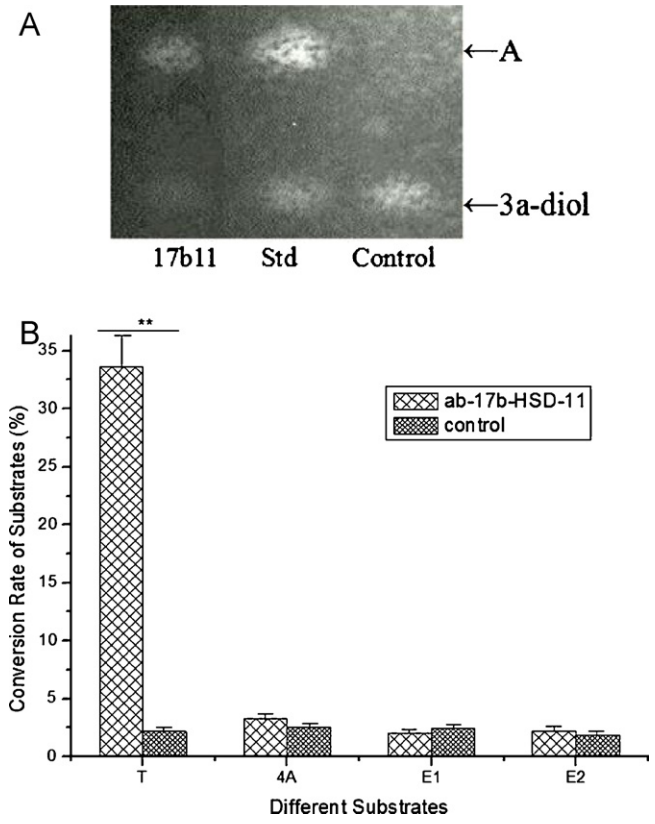

Fig. 2. Enzymatic activity assay of ab-17β-HSD-11 expressed in HEK-293 cells. (A) By thin layer chromatography, conversion of 3α-diol to androsterone. 17β-11, ab-17β-HSD-11 transfected into HEK-293 cells; control, empty vector transfected into HEK-293 cells; Std, standard steroids; 3a-diol, 3α-diol; and A, androsterone. (B) By high performance liquid chromatography, conversion of testosterone, androstenedione, estradiol, or estrone. T, testosterone; 4A, androstenedione; E1, estrone; and E2, estradiol. Results are represented as mean ± SE of three independent measurements.

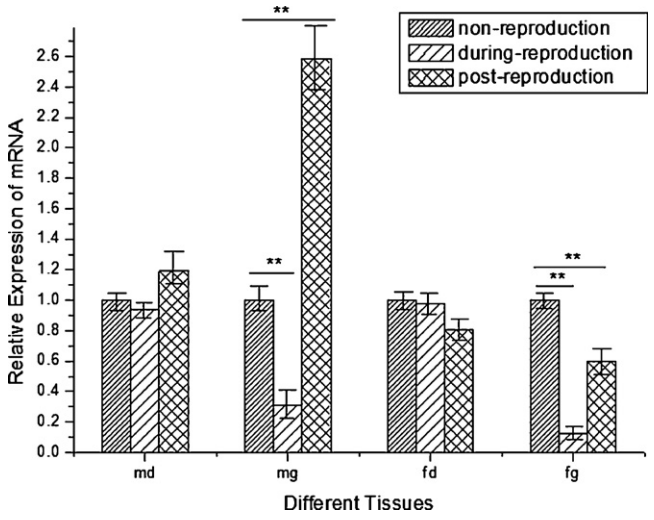

Fig. 3. Quantitative PCR analysis of ab-17β-HSD-11 mRNA expression in various tissues at different reproductive stages. Each column represents an average expression level of three replications. Different tissues: md, digestive gland of male abalone; mg, gonad of male abalone; fd, digestive gland of female abalone; fg, gonad of female abalone. Asterisks indicate significant difference (**$P < 0.01$) using one-way ANOVA by SPSS16.0.

worm) 17β-HSD-11, followed by *Xenopus* (*Silurana*) *tropicalis* (47% identity), and other mammalian species (42–44% identity). Multiple alignments indicated that 17β-HSD-11 sequences are highly conserved in the catalytic domain and the functionally important motifs from the SDR family such as the cofactor-binding domain (TG×××G×G), the catalytic site (Y××SK), and the structural stabilization domain (NNAG) (Supplemental file). Based on alignment of the amino acid sequences of published 17β-HSDs, a phylogenetic tree was constructed using the neighbor-joining algorithm of MEGA 4.1 software. The phylogenetic relationship is shown in Fig. 1. The results indicate that 17β-HSDs are clustered into several clades. 17β-HSD types 1, 2, 6, and 9 form clade 1; types 3, 11, 12, and 13 cluster as clade 2; types 4, 8, 10, and 14 are grouped in clade 3; and types 5 and 7 assemble as clade 4. ab-17β-HSD-11 is located in clade 2: in this clade, types 3 and 12 share a sister branch with types 11 and 13. 17β-HSD types 11 and 13 are intertwined in the phylogenetic tree, which means that they possess an unresolved evolutionary relationship.

3.3. Activity of ab-17β-HSD-11

To further investigate the activity of ab-17β-HSD-11 in processing steroid hormones, a functional analysis was performed by transiently transfecting the ORF of the candidate gene into HEK-293 cells. As illustrated in Fig. 2, ab-17β-HSD-11 predominantly catalyzes oxidative reactions converting 3α-diol and T to A and 4A, respectively. After 23 h ab-17β-HSD-11 had converted 53.4% of 3α-diol to A (Fig. 2A), whereas the conversion rate of T to 4A was about 31.5% at 23 h (Fig. 2B). We also conducted the reverse reactions (*i.e.*, from A to 3α-diol or from 4A to T); unlike the forward reactions, HEK-293 cells transfected with ab-17β-HSD-11 exhibited no obvious catalytic activity for reduction reactions (*i.e.*, ab-17β-HSD-11 could not effectively transform A to 3α-diol or 4A to T) (Fig. 2A and B). It is important also to mention that we did not find any catalytic activity of ab-17β-HSD-11 towards E1/E2, although such reactions have been observed for mammalian 17β-HSD-11 (Fig. 2B).

3.4. Relative mRNA expression of ab-17β-HSD-11

To gain further insight into the putative functions of ab-17β-HSD 11 *in vivo*, its expression pattern was investigated by real-time PCR at different reproductive stages. The results showed that ab-17β-HSD-11 is expressed in both the digestive gland and the gonad tissues (Fig. 3). In both males and females, the relative expression levels of ab-17β-HSD-11 in the digestive glands were not significantly different at different reproductive stages. However, in the gonads, the expression of the ab-17β-HSD-11 gene was different at various reproductive stages. Interestingly, in gonad tissues the expression of ab-17β-HSD-11 decreased significantly during the reproductive period, in contrast to the non-reproductive and post-reproductive periods, but subsequently began to recover during the post-reproductive stage (Fig. 3).

4. Discussion

In this study, we cloned 17β-HSD-11 from abalone, which contains several typical SDR conserved motifs consisting of a cofactor binding domain (TG×××G×G), catalytic activity site (Y××SK), and structural stability motif (NNAG). BLAST analysis revealed that the deduced peptide sequence of ab-17β-HSD-11 shared a high degree of sequence similarity with other 17β-HSD family members, and especially with other 17β-HSD-11s. To further explore whether the putative abalone 17β-HSD-11 is indeed a 17β-HSD-11 homologue, an evolutionary relationship analysis was performed. By collecting some representative members of all published 17β-HSD types (from type 1 to type 14), we constructed a phylogenetic tree. Based on analysis of the phylogenetic tree, the 17β-HSDs were clustered

into several clades. ab-17β-HSD-11 was located in the same branch as other type 11 17β-HSDs in *H. sapiens*, *P. abelii*, *Macaca fascicularis*, *B. Taurus*, *M. musculus*, *Rattus norvegicus*, *Taeniopygia guttata*, *Xenopus* (*Silurana*) *tropicalis*, and *S. kowalevskii* (Fig. 1). In this branch, the closest relationship was between ab-17β-HSD-11 and *S. kowalevskii* 17β-HSD-11, followed by the amphibian and then the mammalian species. This result is consistent with the designation of ab-17β-HSD-11 as a member of the 17β-HSD type 11 family.

It deserves mention that the evolutionary relationship between type 11 and type 13 17β-HSDs was somewhat perplexing since they were intertwined in the phylogenetic tree (Fig. 1). Based on the similarity analysis, 17β-HSD-11 shared high similarity with type 13 17β-HSD. Additionally, it is well known that, in humans, 17β-HSD types 11 and 13 are located on chromosomal position 4q22.1 [9]. Consequently, we can speculate that the two genes originated from a common ancestor and possess different roles through gene duplication and functional divergence. As the biological function of 17β-HSD-13 still remains to be characterized [9,29], we cannot rule out the possibility that types 11 and 13 have the same function although this is subject to proof. In order to determine the exact relationship between the two types of 17β-HSDs, other analyses such as Bayesian and likelihood-based methods could be conducted in the future.

To evaluate the functionality of abalone 17β-HSD-11, its catalytic activity was measured by monitoring the biotransformation of steroids in HEK-293 cells transfected with ab-17β-HSD-11. In the present work, we characterized the conversion of candidate steroids by using TLC and HPLC. Due to its convenience, sensitivity, and low cost, HPLC detection proved to be a very practical tool for detecting low quantities of steroid metabolites [18,30–33]. In view of the fact that 3α-diol and A are difficult to separate through HPLC because of the high similarities of their absorption spectra and retention times, we used TLC to assess the biotransformation of these substrates. The results clearly demonstrate ab-17β-HSD-11 can transform 3α-diol and T to A and 4A, respectively (Fig. 2). To our knowledge, this is the first study to demonstrate the catalytic activity of 17β-HSD-11 in converting androgenic hormones in marine mollusks. The results indicate that ab-17β-HSD-11 is indeed a steroid metabolic enzyme that might be involved in different steroidogenic pathways, depending on its spatial and temporal expression patterns.

A point that should be noted is that neither E1 nor E2 can be transformed by ab-17β-HSD-11, whereas earlier reports showed that 17β-HSD-11 in humans has slight dehydrogenase activity at the 17β position of estradiol [16,18]. We speculate that a difference in three-dimensional structure is possibly the reason for this consequence because we noticed that in ab-17β-HSD-11 the amino acid at position 226 is leucine (L) rather than the corresponding phenylalanine (F) that is present in the other homologous sequences (Supplemental file). Blanchard et al. [34] pointed out that, in mammalian 17β-HSD-12, the bulky amino acid F adjoins the active site and controls the entry of estrogenic substrates, whereas the amino acid L is responsible for the entry of androgenic substrates. Different stereochemical configurations of 17β-HSD-12 create differential substrate specificities for androgens and estrogens. In accord with this opinion, we speculate that in ab-17β-HSD-11 it is the absence of F at site 226 that results in the loss of catalytic activity towards estrogens. However, this tentative proposal needs to be proven by further experiments.

In order to procure more information about the physiological function of ab-17β-HSD-11, we examined mRNA expression levels *in vivo* during different reproductive stages (non-, during-, and post-reproduction). The results show that ab-17β-HSD-11 is expressed in the digestive glands and gonads, which is consistent with published reports [9,17,35]. As there were no obvious differences in expression levels of ab-17β-HSD-11 in digestive gland tissue, we speculate that expression in the digestive gland is independent of reproductive state. In contrast, it is noteworthy that in gonadal tissue, the level of ab-17β-HSD-11 during reproduction in abalones was demonstrably lower than that in the non-reproductive and post-reproductive stages. Since changes in 17β-HSD activity correlate with the reproductive cycles of animals [36], as a consequence, variations in expression of ab-17β-HSD-11 suggest its potential role in regulating abalones' reproductive cycle.Concerning the biological activities of 17β-HSD-11 that are well established, we believe it plays an essential and flexible role in regulating the actions of the steroid hormones, especially androgens. It is well recognized that 17β-HSD-11 can efficiently reduce circulating levels of DHT and T, the most potent androgens, by catalyzing the transformations of DHT to 3α-diol and T to 4A [4,37,38]. Thus, it seems that, in the process of androgen metabolism, 17β-HSD-11 functions as an essential regulator modulating the concentrations of DHT and T, to buffer or mitigate the detrimental impact resulting from excessive active androgens. Therefore, it is reasonable to speculate that ab-17β-HSD-11 likewise functions as a modulator to regulate active steroids, although there is no definitive evidence regarding the presence of DHT or T in abalone at present. In addition, it is not difficult to understand why, in the gonad tissues of abalone during reproduction, the expression of ab-17β-HSD-11 significantly declines because at this stage there is a high demand for active steroids (*e.g.*, DHT, T, or E2) to fulfill relevant physiological roles, which requires tempering of the catabolism of active steroids by ab-17b-HSD-11.

The reasons why there are significant differences between male and female gonads in the expression mode of ab-17β-HSD-11 remain unknown. According to the available data (Fig. 3), we notice that, in both male and female abalone gonads, the expression levels of the target gene follow the same trend of high-low-high during the three reproductive stages. However, in male gonads the expression peak of ab-17β-HSD-11 appears at the post-reproductive stage whereas, in female gonads, it emerges during the non-reproductive phase. Unraveling this conundrum will require us to acquire more knowledge of the role of this gene in abalones' physiological activities.

5. Conclusions

In summary, based on nucleotide and amino acid sequences, we have cloned a 17β-HSD-11 homologue from *H. diversicolor supertexta*. Initial studies were also conducted to characterize the biological activity of ab-17b-HSD-11. Currently available results show that the functional motifs of ab-17β-HSD-11 are endowed with high conservation and an evolutionary relationship with their counterparts in other species. These features indicate that the putative ab-17β-HSD-11 is a potential member of the 17β-HSD subfamily. The conversion capacity (*i.e.*, from 3α-diol and T to A and 4A, respectively) was verified by transiently transfecting the target gene in HEK-293 cells. Moreover, by exploring the expression pattern of ab-17β-HSD-11 *in vivo*, we suggest that in abalone 17β-HSD-11 probably performs an essential function during the steroid-mediated reproductive process. However, this work is preliminary and incomplete; the exact role and subcellular localization of ab-17β-HSD-11 in abalone needs to be further clarified.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.12.013.

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