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Molecular characterization and quantification of the follicle-stimulating hormone receptor in turbot (*Scophthalmus maximus*)

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Abstract Molecular cloning, characterization, and functional analysis of follicle-stimulating hormone receptor (FSHR) in female turbot (*Scophthalmus maximus*) were evaluated. Results showed that the full-length FSHR cDNA was 3824 bp long and contained a 2202 bp open reading frame that encoded a mature protein of 733 amino acids (aa) and a signal peptide of 18 aa. Multiple sequence analyses showed that turbot FSHR has high homology with the corresponding genes of other teleosts and significant homology with that of *Hippoglossus hippoglossus*. Turbot FSHR has the typical structural architecture of glycoprotein hormone receptors consisting of a large N-terminal extracellular domain, seven transmembrane domains and short C-terminal intracellular domain. FSHR mRNA was found to be abundant in the ovaries, but deficient in eyes, intestine, brain, muscle, gills, spleen, stomach, heart and kidney. Furthermore, FSHR mRNA was found to increase gradually from pre-vitellogenesis to migratory nucleus stages, with the highest values observed during the late vitellogenesis stage of the reproductive cycle. However, FSHR mRNA was found to decrease dramatically during the atresia stage. Meanwhile, functional analysis with HEK293T cells continual expressing FSHR demonstrated that FSHR was specifically stimulated by ovine FSH, but not ovine LH. These results indicate that turbot FSHR is mainly involved in the stimulation of vitellogenesis, regulation of oocyte maturation as well as promotion of ovarian development via specific ligand binding. These findings open doors to further investigation of physiological functions of FSHR, which will be valuable for fish reproduction and broodstock management.

Keywords RACE · Quantitative real-time PCR · Follicle-stimulating hormone receptor · Reproduction · Turbot (*Scophthalmus maximus*)

Yudong Jia and Ai Sun have equal contribution to this work.

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Introduction

The development and maturation of ovarian oocytes are key physiological processes in female reproduction. Oogenesis is strictly regulated by numerous

endocrine and paracrine factors, particularly gonadotropic hormones (Hurk and Zhao 2005; Lubzens et al. 2010; Chaffin and Vandevoort 2013). In teleosts, like in other vertebrates, gonadotropins mainly act on gonadal tissue by binding to specific receptors, namely follicle-stimulating hormone receptor (FSHR, formerly termed GtHR-I) and luteinizing hormone receptor (LHR, formerly termed GtH-RII) (Swanson et al. 2003; Levavi-Sivan et al. 2010). These receptors are primarily expressed on the surfaces of gonadal somatic cells, and involved in the regulation of gametogenesis via distinct intracellular signaling pathways (Menon and Menon 2012). Numerous studies have reported that fish gonadotropin receptors (GtHRs) exhibit typical characteristics of a glycoprotein hormone receptor and belong to the G protein-coupled receptors (GPCR) superfamily, which are complex transmembrane (TM) rhodopsin-like proteins characterized by seven hydrophobic helices inserted in the plasmalemma as well as intracellular and extracellular domains (Kumar et al. 2001a, b; Oba et al. 2001; Kwok et al. 2005; Rocha et al. 2007; Levavi-Sivan et al. 2010). However, ligand specificity of fish GtHRs is not as well defined as it is in mammalian species and cross-activation may occur under physiological conditions (Bogerd 2007; Levavi-Sivan et al. 2010). Two genetically distinct GtHRs have been documented in amago salmon (Oba et al. 1999a, b), African catfish (Vischer and Bogerd 2003), Atlantic salmon (Maugars and Schmitz 2006), European sea bass (Rocha et al. 2007) Atlantic halibut (Kobayashi and Andersen 2008), Atlantic cod (Mittelholzer et al. 2009), and mummichog (Ohkubo et al. 2013). In addition, molecular biological studies have shown that the two receptors have different expression profiles in repetitive and annual spawning fish during the reproductive cycle (Kumar et al. 2001a, b; Kwok et al. 2005; Kobayashi et al. 2008; Andersson et al. 2013). Thus, detailed information about the characteristics, function and regulation of GtHRs is necessary for sound understanding of potential physiological functions in fish reproductive cycle, which may be useful in controlling fish reproduction and improving broodstock management.

Turbot (*Scophthalmus maximus*), which has high economic value, delicious meat and rapid growth, is cultured widely in Europe and Asia. Some preliminary studies have been conducted on the nutritional requirements, hormonal induction of spawning, egg quality, and the effect of environmental conditions on female turbot during its reproductive cycle (Jones 1974; Mcevoy 1989; Suquet et al. 1995; Bromley et al. 2000; Jia et al. 2014a). Mature female turbot can spawn 8–12 times at intervals of 3–5 days during the spawning season (Mugnier et al. 2000). The follicles in the mature female turbot ovary represent different developmental stages throughout spawning season. Numerous genes were confirmed to be related to reproduction and involved in sex differentiation in turbot (Ribas et al. 2013). However, the molecular mechanisms that regulate asynchronous development of multiple batches of oocytes in turbot and the functional properties of GtHRs in the ovary during the reproductive cycle remain poorly understood. The related study has been conducted to elucidate the molecular cloning, molecular characteristics and temporal expression profiles of turbot LHR (Jia et al. 2014b). In the present study, we sought to investigate GtHRs in turbot in great detail by cloning and characterizing FSHR from turbot ovarian tissues. In addition, FSHR expression profiles and functions of turbot during the reproductive cycle were evaluated.

Materials and methods

Fish and tissue sampling

Sexually mature female turbots were obtained from Tianyuan Fisheries Co., Ltd. (Shandong Province, China). The turbots were rapidly removed after anesthetized with 100 mg/L tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO, USA). Tissues samples (ovary, eye, intestine, brain, muscle, ovary, gill, spleen, liver, stomach, heart and kidney) were collected from each fish and stored in liquid nitrogen for RNA extraction. The ovaries were placed in Bouin's solution for hematoxylin and eosin staining to identify the oocyte developmental stages according to our previous study (Jia et al. 2014b). The stages of ovarian development are as follows: pre-vitellogenesis (Prevtg), early vitellogenesis (Evtg), late vitellogenesis (Latvtg), migratory-nucleus (Mig-nucl) and atresia (Atre). Meanwhile, testis were collected from six mature male turbots and stored in liquid nitrogen for RNA extraction.

FSHR gene cloning

Total RNA was extracted using Trizol (Invitrogen) from turbot ovaries according to the manufacturer's protocol. Quality and quantity of total RNA were analyzed by 1 % agarose gel electrophoresis and quantified by UV spectrophotometry. To obtain full-length 5′- and 3′-termini of the FSHR gene, the SMART^{TM} RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) was used. Approximately 200 ng of total RNA was used to synthesize 5′- and 3′-RACE-Ready cDNA based on the manufacture's instruction. Two primers (FSHRF0 and FSHRR0) were designed according to conserved sequences of the FSHR gene from other teleost species to obtain a partial fragment of turbot FSHR cDNA. Based on this partial nucleotide sequence of FSHR, two specific primers (FSHRGSP1 and FSHRGSP2) were designed for 5′- and 3′-RACE using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). The universal primer (UPM) used for 5′- and 3′-RACE was a mixture of long and short primers (supplied with SMART^{TM} RACE cDNA Amplification Kit, Clontech). The primers mentioned above are listed in Table 1.

Sequence analysis

DNA sequence data were edited and analyzed using the open reading frame (ORF) finder available at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to deduce the translation initiation site (ATG) and the primary structure of FSHR. The structural features of FSHR were searched using TMHMM Server 2.0 (http://www.cbs.dtu.dk/services//TMHMM-2.0/). For signal sequence prediction, the SignalP program was used (http://www.cbs.dtu.dk/services/SignalP/). Prediction of potential N-linked glycosylation sites and phosphorylation sites were carried out using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetPhos (http://www.cbs.dtu.dk/services/NetPhos/), respectively. Multiple alignments of amino acid sequences were performed using the software ClustalX 1.81. The neighbor-joining (NJ) phylogenetic tree was constructed using MEGA4.0. Sea lamprey (*Petromyzon marinus*) glycoprotein hormone receptor I and II were used as the out-group. Reliability of the NJ tree was assessed by interior branch test, using 1000 replications. The deduced amino acid sequences of vertebrate FSHR were acquired from the GenBank database.

Functional analysis

For functional analysis of cloned turbot FSHR, we directly amplified the ORF of FSHR using the primers FSHR–EcoRI and FSHR–BamHI (Table 1). The amplified cDNA were then digested by EcoRI and BamHI, and the digested fragments were ligated into the EcoRI/BamHI site of pEGFP-N3 vector (BD Biosciences Clontech, USA). Then, HEK293T cells were transfected with the pEGFP-N3-FSHR plasmid

Table 1 Primer sequences used in this study

Primers	Primer sequence 5′–3′	Purpose used
FSHRF0	AGCCGCTCCAAACTGACTGGAG	cDNA fragment of FSHR
FSHRR0	TGAGGTAACAGCCACACATGAG	cDNA fragment of FSHR
Long primer	CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT	RACE
Short primer	CTAATACGACTCACTATAGGGC	RACE
FSHRGSP1	CAGGGTGATCGCTGTTAATGTG	5′-RACE
FSHRGSP2	TGGATCTTCTCATCTCTTGCCG	3′-RACE
FSHRF	CGTATCAAAGTCGCAAGAA	qRT-PCR
FSHRR	TGCAGTTCGACAATAAAGT	qRT-PCR
β-actinF	TGAACCCCAAAGCCAACAGG	qRT-PCR
β-actinR	CAGAGGCATACAGGGACAGCAC	qRT-PCR
FSHR–EcoRI	GGTGAATTCATGATGATGAGTCTGACGGT	Functional analysis
FSHR–BamHI	GGTGGATCCAAACGCTACAGGTTGTTTT	Functional analysis

using Lipofectamine® 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, Hyclone, New Zealand), penicillin (100 Units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5 % CO_2 at 37 °C. The continual expressing pEGFP-N3-FSHR HEK293 cells were established in DMEM containing 10 % FBS and 100 μg/ml of G-418 (Roche). The transcripts in the transfected cells were confirmed by observing green fluoresce and RT-PCR after transfection for 24 h. Subsequently, cells were seeded in 24-well culture plates (Nunc, Denmark) at a density of 1.4×10^5/well in DMEM supplemented with 0.1 % FBS and stimulated by adding the specified amounts of hormones in the growth medium for the indicated times. Cells were treated with ovine FSH (OVAGENTM, New Zealand) at 0.1–1000 ng/ml and ovine LH (OVAGENTM, New Zealand) at 0.1–1000 ng/ml for 12 h. Cell lysis and measurement of intracellular cAMP was carried out using the cAMP enzyme immunoassay system Kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. All measurements were taken in triplicate. The chemicals were dissolved in ethanol or dimethylsulfoxide and then diluted with media. The concentration of ethanol or dimethylsulfoxide in the medium was ≤0.1 %. Control cultures received only vehicle.

Real-time quantitative reverse transcription PCR (qRT-PCR)

Real-time qRT-PCR was performed to determine the relative expression of FSHR mRNA in tissues and the different oocyte developmental stages. Briefly, total RNA was extracted from the collected tissues with Trizol (Invitrogen) and measured using NanoDrop 2000 (Thermo fisher scientific, Rockford, IL, USA). Total RNA was reverse transcribed using a Thermo Fish One step RT-PCR kit according to the manufacturer's instructions. Levels of FSHR and β-actin mRNAs were determined by real-time RT-PCR using TaKaRa RT-PCR Master Mix reagent and ABI StepOne Plus Sequence Detection System (Applied Biosystems, USA). The SYBR Premix Ex Taq™ Kit (Takara Bio., China) was used for amplification, and the reaction mixture composition was as follows: 10 μL of SYBR® Premix Ex Taq™, 0.8 μL of each primer (10 μM), 0.4 μL of ROX Dye (50×), μL of cDNA sample (25 ng/μL) and 6 μL of sterile distilled water. Initial denaturation was conducted at 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 30 s. The primers FSHRF/R and β-actinF/R were used to amplify the FSHR and β-actin fragments, respectively (Table 1). Two genes displayed the same amplification efficiency via assessing the standard curve by real-time PCR (Supplemental data, S1). All samples were amplified in triplicates. The relative abundance of FSHR mRNA was normalized to β-actin according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Fig. 1 Nucleotide (*upper line*) and deduced amino acid (*lower line*) sequences of the turbot FSHR. The start codon (ATG) was *boxed*, and the stop codon (TGA) was marked with an *asterisk*. The signal peptide sequence and the polyA signal in 3′-UTR are underlined. The position of the seven predicted transmembrane domain is showed as *black box*. Two conserved cysteins (^{477}C, and ^{552}C), predicted to form an intramolecular disulfide bond, are indicated by *dots*. Potential N-glycosylation and phosphorylation sites are indicated by *open* and *closed triangles*

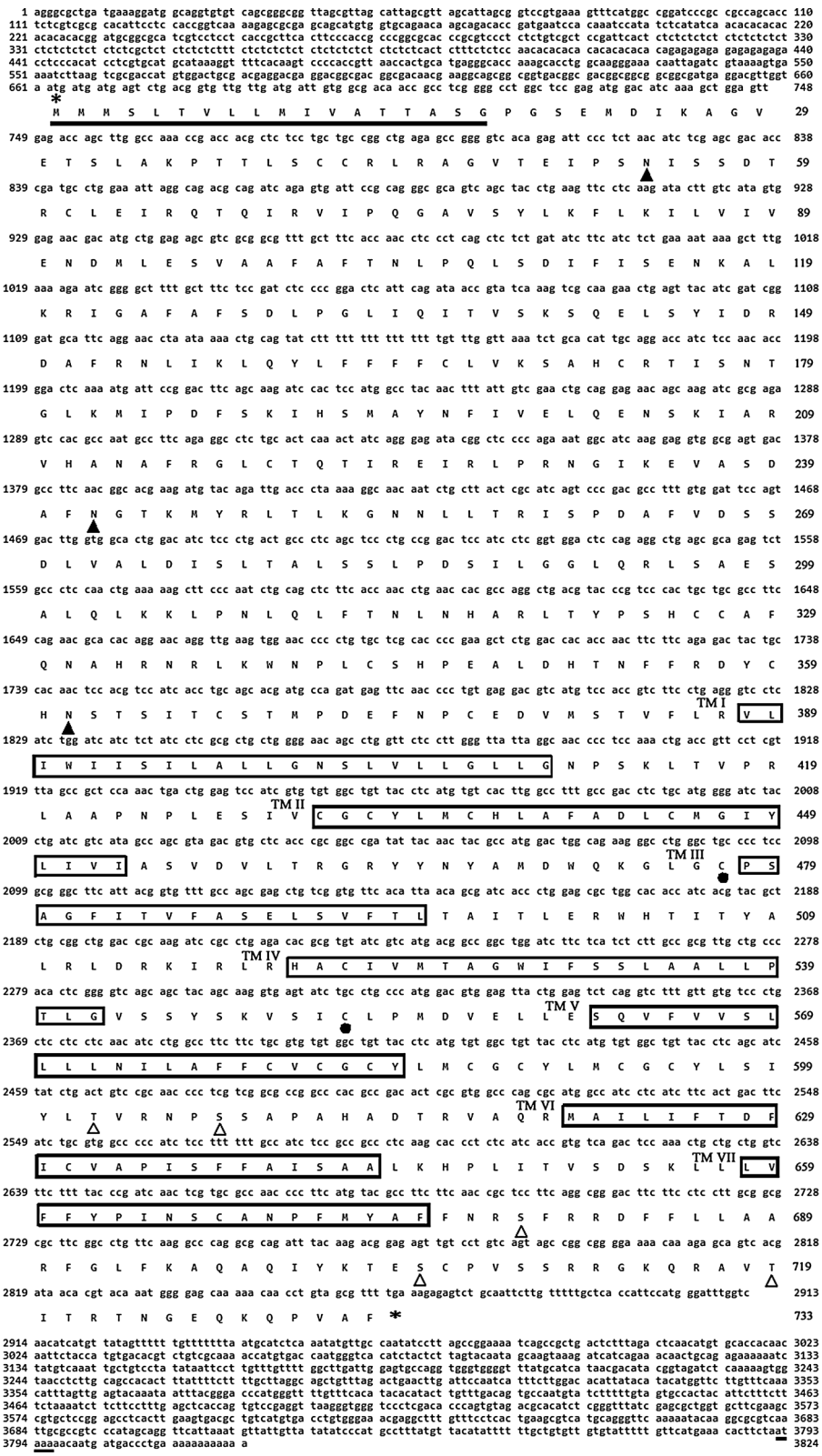
Statistical analysis

The experiment was repeated at least three times for each condition. All data were expressed as mean ± SEM and analyzed by ANOVA and Duncan's multiple range tests using the SAS 8.0 software. $P < 0.05$ was considered to be statistically different.

Results

Cloning and characteristics of FSHR gene from turbot

The full-length cDNA sequence of FSHR was obtained by 5′- and 3′-RACE. The FSHR cDNA was 3824 bp long with a 2202 bp ORF coding 733 residues of the mature protein, and 661 bp of 5′ and 961 bp of 3′ terminal untranslated regions (UTRs). The 3′-UTR contained one canonical polyadenylation signal (AATAAA), followed by an additional 12 bp in the poly (A) tail (Fig. 1). Structural analyses revealed that turbot FSHR exhibits typical characteristics of a glycoprotein hormone receptor, which is a subfamily


TM I
TM II
TM III
TM IV
TM V
TM VI
TM VII

of GPCRs. The turbot FSHR has a large extracellular domain with 387 amino acids, including a putative signal peptide of 18 amino acids, three potential N-linked glycosylation sites, flexible hinge region, and seven-transmembrane (TM) domain upstream of the intracellular C-terminus (Fig. 1). The extracellular loops between TM II–III and TM IV–V each contains a cysteine residue (Cys477 and Cys552) that presumably links the extracellular loops via a disulfide bridge (Fig. 1). The intracellular loops between TM V–VI and the intracellular C-terminal domain contain serine and threonine residues that are potential phosphorylation sites (Fig. 1).

Phylogenetic analysis and alignment

Comparison of the turbot FSHR amino acid sequence with teleost, mammalian and other vertebrates showed that turbot FSHR has high homologies with FSHRs of other fish species, followed by mouse, chicken and frog FSHRs (Fig. 2). In addition, turbot FSHR was found to contain three specific insertions (aa 162–174, 421–434, 580–592) that are not present in other pleuronectiformes (Fig. 2). Comparison with the paralogous genes LHR and thyroid stimulating hormone receptor (TSHR) demonstrated that the turbot FSHR protein is closely related to *Hippoglossus hippoglossus* proteins, as depicted in the phylogenetic tree in Fig. 3.

Tissue distribution of the gene expression of FSHR

The tissue distribution of FSHR was analyzed by qRT-PCR. FSHR mRNA was found to be significantly most abundant in the ovary (Fig. 4a, $P < 0.05$) followed by the liver. Expression of the FSHR was also detected in several extra-gonadal tissues such as the eyes, intestine, brain, muscle, gills, spleen, stomach, heart and kidney at significantly lower levels than the ovary (Fig. 4a, $P < 0.05$). However, FSHR mRNA was not found to be significantly different among these extra-gonadal organs (Fig. 4a, $P > 0.05$). FSHR mRNA was also detected in testis, albeit at significantly lower levers compared to the ovary (Fig. 4b, $P < 0.05$). Furthermore, the tissue distribution of FSHR mRNA was found to be similar to that of LHR mRNA in turbot (supplemental data, S2A). However, FSHR mRNA was significantly higher than LHR mRNA in the ovary and the liver (supplemental data, S2A).

Fig. 2 Alignment of the amino acid sequence of the turbot FSHR (SM-FSHR) proteins from various species. Position with >70 % similarity are shaded in *light gray*, while completely conserved positions are shaded in *black*. Accessions number: *Cynoglossus semilaevis* (CS) ACD39387.2, *Epinephelus coioides* (EC) AEG65826.1, *Hippoglossus hippoglossus* (HH) ACB13177.1, *Solea senegalensis* (SS) ADH51678.1, *Clarias gariepinus* (CG) AJ012647.2, *Danio rerio* (Z) AAP33512.1, *Xenopus laevis* (XL) NM_001256260.1, *Gallus gallus* (GG) NM_205079.1, *Mus musculus* (M) NM_013523.3, *Humo sapiens* (H) M65085.1

The expression profile of the FSHR gene during ovarian developmental stages

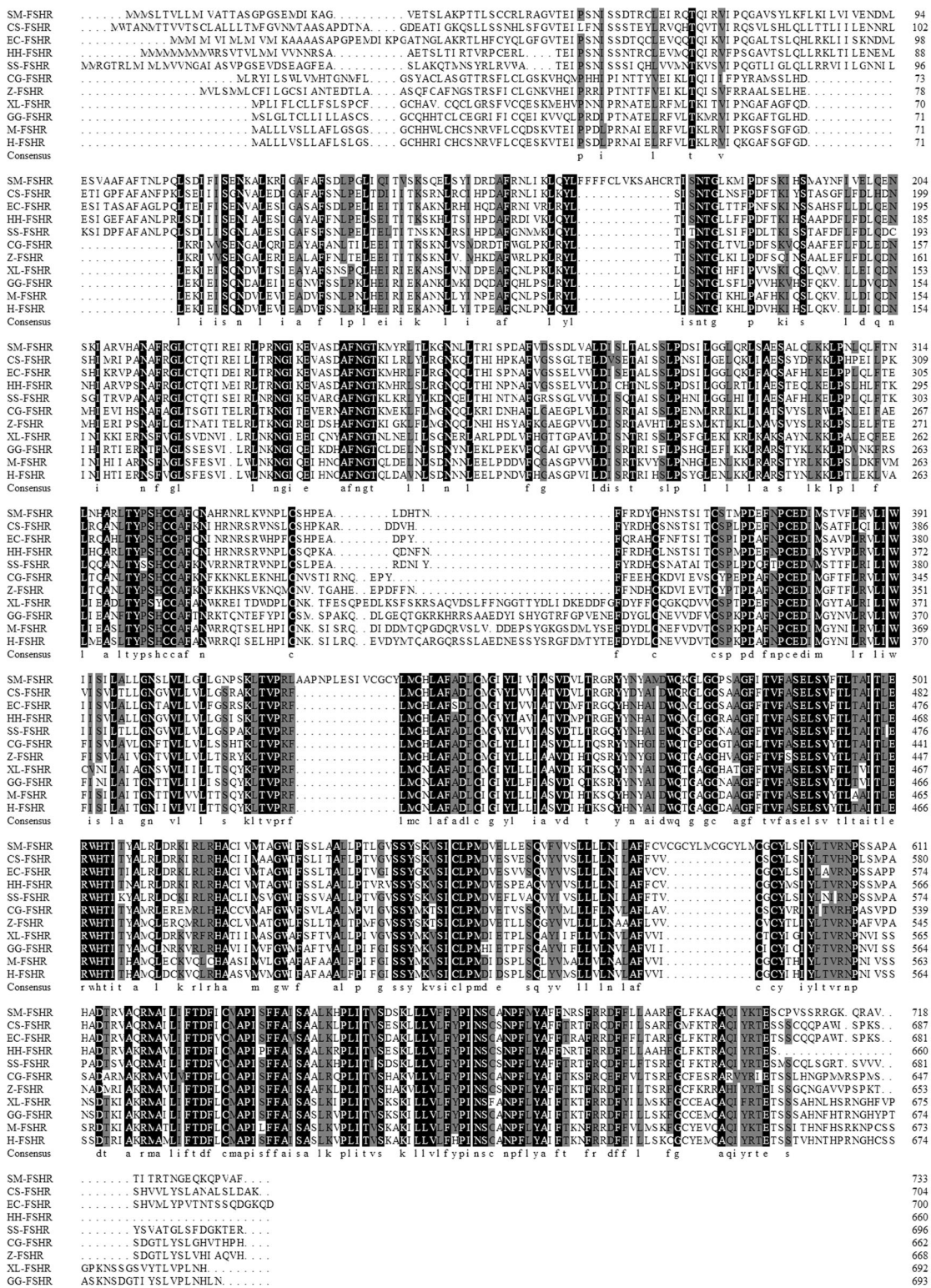
Gene expression profiles of FSHR at distinct developmental stages of the ovary were examined by qRT-PCR. FSHR mRNA gradually increase from the Prevtg to the Mig-nucl stage, with the highest values observed during the Latvtg stage (Fig. 5, $P < 0.05$). However, FSHR mRNA decreased dramatically during the Atre stage (Fig. 5, $P < 0.05$). Meanwhile, the highest values of FSHR mRNA and LHR mRNA were obtained in the Latvtg and Mig-nucl stages, respectively (supplemental data, S2B).

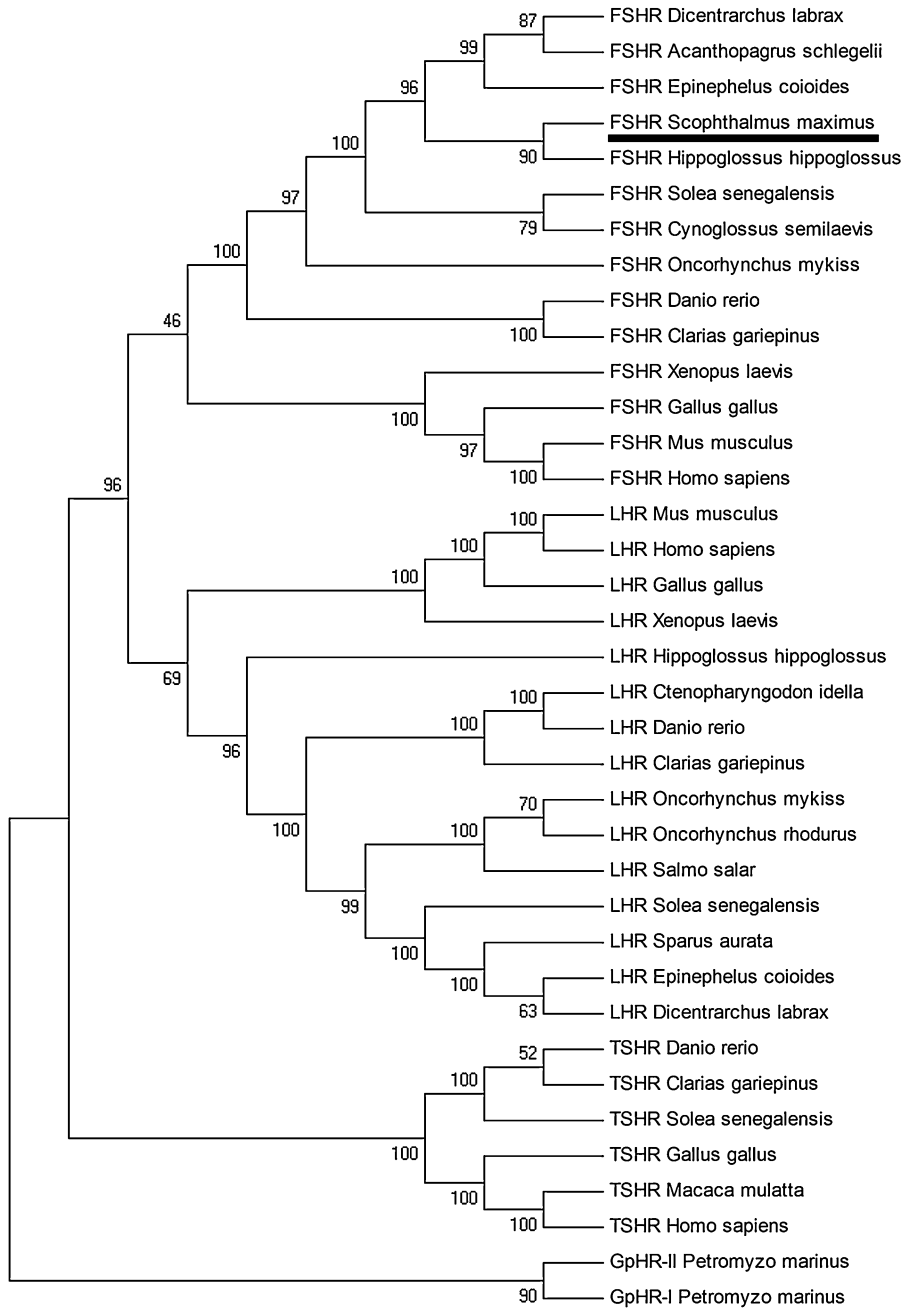
Functional analysis of the FSHR

HEK293T cells constantly expressed turbot FSHR after transfection with the pEGFP-N3-FSHR plasmid (Fig. 6). The HEK293T cells can continually express turbot FSHR after the transfection for 24 h (supplemental data, S3). Then, the transfected HEK293T cells were treated with ovine FSH (0.1–1000 ng/ml) and ovine LH (0.1–1000 ng/ml) for 12 h. After treatment with ovine FSH, intercellular cAMP level of HEK293T cells continual expressing FSHR evidently increased in a dose-dependent manner from 0.1 to 100 ng/ml, but decreased at 1000 ng/ml (Fig. 7a, $P < 0.05$). However, the intercellular cAMP level of HEK293T cells continual expressing FSHR did not change upon treatment with ovine LH at all concentrations tested (0.1–1000 ng/ml; Fig. 7b, $P > 0.05$).

Discussion

As the most diverse group of vertebrates, fish have various modes of ovarian development and reproductive strategies (Nagahama and Yamashita 2008). The


SM-FSHR
CS-FSHR
EC-FSHR
HH-FSHR
SS-FSHR
CG-FSHR
Z-FSHR
XL-FSHR
GG-FSHR
M-FSHR
H-FSHR
Consensus



physiological function of gonadotropins and their receptors during the reproductive cycle in teleost is highly controversial (Levavi-sivan et al. 2010). In present study, the FSHR cDNA of turbot was cloned from ovarian tissue and its structural characteristics and expression profiles were evaluated. Analysis of the nucleotide and deduced amino acid sequence of turbot FSHR revealed that this receptor is highly homologous to teleost FSHRs and shows typical structural features of glycoprotein hormone receptors. A relatively long extracellular domain and rhodopsin-like seven TM module were observed. Meanwhile, a highly conserved amino acid sequence (YPSHCCAF) proposed to form a pocket for specific glycoprotein

◀**Fig. 3** The neighbor-joining phylogenetic tree of vertebrates based on the amino acid sequences of TSHR, FSHR and LHR. Accession number: FSHR *Acanthopagrus schlegelii* (ABU49599.1), FSHR *Dicentrarchus labrax* (AY642113.1), FSHR *Epinephelus coioides* (AEG65826.1), FSHR *Hippoglossus hippoglossus* (ACB13177.1), FSHR *Solea senegalensis* (ADH51678.1), FSHR *Cynoglossus semilaevis* (ACD39387.2), FSHR *Oncorhynchus mykiss* (AF439405.1), FSHR *Clarias gariepinus* (AJ012647.2), FSHR *Danio rerio* (AAP33512.1), FSHR *Xenopus laevis* (NM_001256260.1), FSHR *Gallus gallus* (NM_205079.1), FSHR *Humo sapiens* (M65085.1), FSHR *Mus musculus* (NM_013523.3), TSHR *Danio rerio* (NM_001145763.2), TSHR Homo sapiens (AY429111.1), TSHR *Gallus gallus* (NM_001193589.1), TSHR *Macaca mulatta* (NM_001195395.1), TSHR *Clarias gariepinus* (AY129556.1), TSHR *Solea senegalensis* (FN677495.1), LHR *Xenopus laevis* (AB602929), LHR *Gallus gallus* (AB009283), LHR *Homo sapiens* (S57793), LHR *Mus musculus* (M81310), LHR *Hippoglossus hippoglossus* (EU502845), LHR *Danio rerio* (AY714133), LHR *Ctenopharyngodon idella* (EF194761), LHR *Clarias gariepinus* (AF324540), LHR *Oncorhynchus rhodurus* (AB030005), LHR *Oncorhynchus mykiss* (AF439404), LHR *Solea senegalensis* (GQ472140), LHR *Salmo salar* (AJ579790), LHR *Sparus aurata* (AY587261), LHR *Dicentrarchus labrax* (AY642114), LHR *Epinephelus coioides* (HQ650770). Sea lamprey (*Petromyzo marinus*) glycoprotein hormone receptor (GpHR) I (AY750689) and GpHR II (AY750689) were used as the out-groups. Bootstrap values (in %) from 1000 replicates are indicated for each tree node

hormone binding and located at the C-terminal exon that forms the extracellular domain of mammalian GtHRs (Lloyd and Griswold 1995) was found to be entirely conserved within the turbot FSHR amino acid sequence (322–329). Cys477 and Cys552 were found to be located in the extracellular loops I and II of the TM domain and were believed to connect the two loops via a disulfide bridge (Levavi-Sivan et al. 2010). Three specific amino acid sequences (162–174, 421–434, 580–592) in the ligand-binding domain

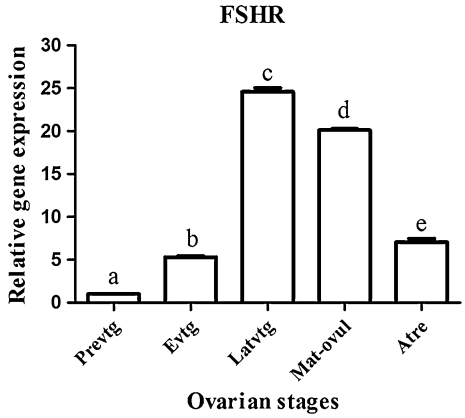


Fig. 5 Quantitative analyses of FSHR mRNA expression in ovarian developmental stages of turbot. Values represent the mean ± SEM ($n = 6$). *Bars* with different superscripts are statistically different ($P < 0.05$)

and were found to be unique in turbot compared to other pleuronectiformes. Moreover, three potential N-linked glycosylation sites and five phosphorylation sites were identified in the N-terminal and C-terminal regions of turbot FSHRs, respectively. These structural features of FSHR are crucial determinants of ligand binding affinity and signal transduction and play important roles in hormone recognition and receptor activation (Moyle et al. 2004; Vassart et al. 2004; Kene et al. 2005; Ulloa-Aguirre et al. 2007; Jiang et al. 2014). Furthermore, phylogenetic analysis showed that turbot FSHR has significant homology with *H. hippoglossus* FSHR. These results indicated that the basic structure and characteristics of turbot FSHR are similar to those of other fish and mammalian species and provide valuable insights into the structural and functional evolution of GtHRs in fish and more generally in vertebrates.

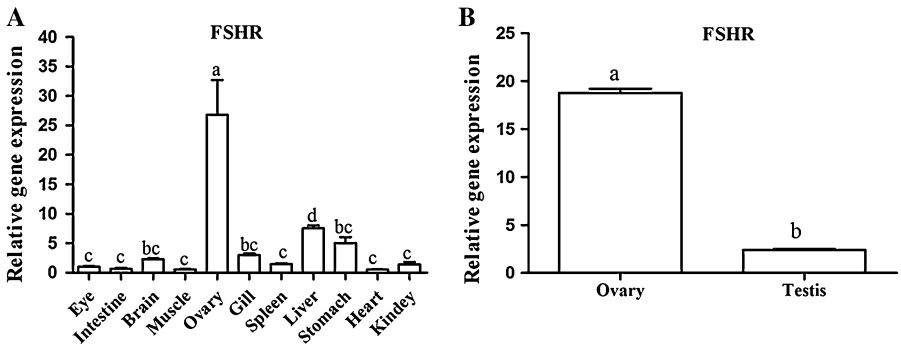


Fig. 4 Quantitative analyses of FSHR mRNA expression in different tissues of turbot. Values represent the mean ± SEM ($n = 6$). *Bars* with different superscripts are statistically different ($P < 0.05$)

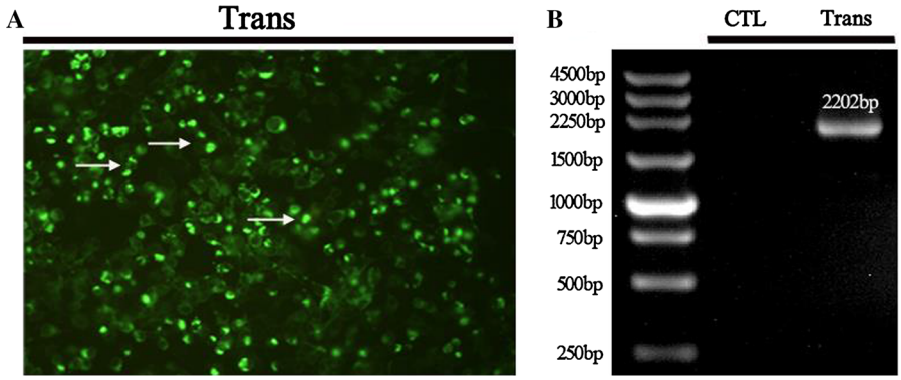


Fig. 6 FSHR was transfected into HEK293T cells by PEGFP-N3 vector. **a** The expression of GFP gene in HEK293T cells after transfected 24 h. **b** PCR-detection of FSHR in transfected HEK293T cells. GFP-labeled HEK293T cells are denoted by *arrows*. *Trans* transfect; *CTL* control

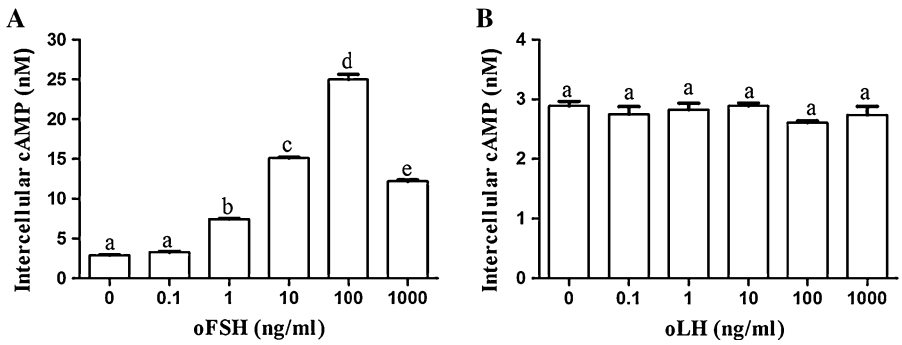


Fig. 7 Functional analysis of FSHR. HEK293T cells expressed the FSHR were treated by ovine FSH (**a**) and ovine LH (**b**), respectively. Values represent the mean ± SEM ($n = 5$). *Bars* with different superscripts are statistically different ($P < 0.05$)

FSHRs are mainly expressed on the surface of ovarian cells and involved in mediating ovarian steroidogenesis, vitellogenesis and may affect follicular development via complex signaling pathways in fish and mammals (Clelland and Peng 2009; Luckenbach et al. 2011; Liu and Ge 2013; Lan et al. 2014; Chakraborty and Roy 2015). Meanwhile, FSHRs are also found in extragonadal tissues, including the reproductive tract, placenta, umbilical cord and brain (Ziecik et al. 1992; Liang et al. 2012). In the present study, turbot FSHR was found to be expressed more abundantly in the ovaries than in other tissues, which is in agreement with reports on other fish species (Oba et al. 1999a; Kumar et al. 2001b; Kwok et al. 2005; Maugars and Schmitz 2006; Rocha et al. 2007; Kobayashi et al. 2008; Ohkubo et al. 2013). However, extragonadal expression of FSHR in turbot was found to be different from that in several other fish species. No extragonadal expression of FSHR has been found in Atlantic salmon (Maugars and Schmitz 2006) and sea bass (Rocha et al. 2007), while remarkable expression of FSHR in the pituitary has been reported in the Atlantic halibut (Kobayashi et al. 2008). The difference in FSHR expression levels among extragonadal tissues may be species-specific and should be investigated further along with the in vivo functions of the gene. It is noteworthy in this context that the results of the present study suggest that turbot FSHR is associated with ovarian development and may have a function in female turbot reproductive cycle.

Oocyte development is triggered by gonadotropins, and the subsequent steroidogenic production of estradiol induces vitellogenesis, causing a marked enlargement in oocyte size (Hillier 2001). Meanwhile, gonadotropins are involved in the regulation of oocyte meiotic resumption and oocyte maturation (Fortune 1994). Complex hormonal cross-talk between the developing oocyte and its surrounding follicular cells has been observed (Canipari 2000; Magoffin 2005). Most teleost fish species are oviparous and have three main types of ovarian development based on oocyte development (synchronous, group-synchronic and asynchronous) (Wallace and Selman 1981). Therefore, the regulatory mechanisms of oocyte development and reproductive strategies may differ among different fish species. Turbot is a serial spawner,

producing multiple egg batches during reproductive season. Histological observations of the turbot ovary revealed that oocyte maturation involved a vitellogenesis step before the oocyte maturation and ovulation stages in our previous study (Jia et al. 2014b). In the current study, FSHR expression was found to increase from previtellogenic to migratory nucleus stages, and the highest expression level was observed at the oocyte late vitellogenic stage. Meanwhile, we also found the FSHR mainly located on the theca cell in vitellogenic follicles of turbot (Data not shown). Studies on multiple and group-synchronous spawners demonstrated that FSHR is mostly correlated with different phases of follicular development and predominantly associated with vitellogenesis (Hirai et al. 2002; Kwok et al. 2005). Similar results were observed in European sea bass (Rocha et al. 2009). These results suggest that FSHR is involved in the stimulation of vitellogenesis and oocyte maturation, thereby promoting ovarian development of turbot during its reproductive cycle.

In mammals, gonadotropin hormone/receptor interactions are very specific, with no cross-stimulation occurring under physiological conditions. However, promiscuous activation of fish GtHRs has been described in salmonids, catfish and other teleosts (Oba et al. 1999a,b; Kumar et al. 2001a,b; Vischer and Bogerd 2003; Kwok et al. 2005; Bogerd 2007). In the present study, turbot FSHR displayed ligand selectivity as it was only activated by ovine FSH, but not by ovine LH. Strict ligand selectively has also been reported in several other species such as rainbow trout (Sambroni et al. 2007), sea bass (Molés et al. 2011), mummichog (Ohkubo et al. 2013), and chub mackerel (Nyuji et al. 2013). Similar results have been observed in zebrafish and amago salmon (Kwok et al. 2005; Oba et al. 1999b). Nevertheless, mammalian hormones can act in a different way than other fish GtHRs (Bogerd 2007; Levavi-Sivan et al. 2010). This difference in ligand selectivity could suggest that the action of GtHRs in teleosts does not overlap fully with that of their mammalian counterparts, and underscores the need for learning more from turbot and other fish.

In conclusion, full-length cDNAs coding for FSHR were cloned from turbot ovaries. Cloning and structural characterization of FSHR provided new insights into the conservation of glycoprotein hormone receptors among vertebrate. Furthermore, our data on the distribution and expression profiles of FSHR are consistent with its involvement in vitellogenesis and oocyte maturation of turbot. In addition, functional analysis with HEK293T cells continual expressing FSHR demonstrated that FSHR is specifically stimulated by ovine FSH, but not ovine LH. These results strongly suggest that FSHR may have crucial functions in regulating turbot ovarian and follicular development. However, additional investigation using recombinant turbot LH and FSH is essential to determine the spatiotemporal expression patterns and ligand availability of LHR and FSHR which will be helpful in further understanding the functions of GtHRs in regulating the reproductive cycle in fish.

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