

Molecular characterization and quantification of the folliclestimulating 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

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

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Y. Jia · A. Sun · Z. Meng · B. Liu · J. Lei Qingdao Key Laboratory for Marine Fish Breeding and Biotechnology, Qingdao 266071, People's Republic of China 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*)

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 proteincoupled 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 necessarv 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 fulllength 5'- and 3'-termini of the FSHR gene, the SMARTTM 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 SMARTTM 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 SMARTTM 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)

Table 1 Primer sequences used in this study

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

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 μ g/ml) in a humidified atmosphere of 5 % CO₂ 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 (OVA-GENTM, 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 TaqTM Kit (Takara Bio., China) was used for amplification, and the reaction mixture composition was as follows:

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*

10 μ L of SYBR[®] Premix Ex TaqTM, 0.8 μ L of each primer (10 μ M), 0.4 μ L of ROX Dye (50×), μ L of cDNA sample (25 ng/ μ L) and 6 μ L 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^{-ΔΔCT} method (Livak and Schmittgen 2001).

Statistical analysis

The experiment was repeated at least three times for each condition. All data were expressed as mean \pm 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

660 748 661 a atg atg atg agt ctg acg gtg ttg ttg atg att gtg gcg aca acc gcc tcg ggg cct ggc tcc gag atg gac atc aaa gct gga gtt MMSLTVLLMIVATTASGPGSEMDIKAGV M 29 749 gag acc agc ttg gcc aaa ccg acc acg ctc tcc tgc tgc cgg ctg aga gcc ggg gtc aca gag att ccc tct aac atc tcg agc gac acc 838 ETSLAKPTTLSCCRLRAGVTEIPSNISSD т 59 839 cga tgc ctg gaa att agg cag acg cag att aga gtg att ccg cag ggc gca gtc agc tac ctg aag ttc ctc aag ata ctt gtc ata gtg 928 R C L E I R O T O I R V I P O G A V S Y L K F L K I L V I V 89 929 gag aac gac atg ctg gag agc gtc gcg gcg ttt gct ttc acc aac ctc cct cag ctc tct gat atc ttc atc tct gaa aat aaa gct ttg 1018 M L E S V A A F A F T N L P O L SDIF I s F N K L 119 atc ggg gct ttt gct ttc tcc gat ctc ccc gga ctc att cag ata acc gta tca aag tcg caa gaa ctg agt tac atc gat cgg A F A F S D L P G L I Q I T V S K S ΙG OELS Y I D R 149 1109 gat gca ttc agg aac cta ata aaa ctg cag tat ctt ttt ttt ttt ttt ttt ttg ttg gtt aaa tct gca cat tgc agg acc atc tcc aac acc 1198 R N L I K L Q Y L F F F F C L V K S A нсвт D A F I s N т 179 1199 gga ctc aaa atg att ccg gac ttc agc aag atc cac tcc atg gcc tac aac ttt att gtc gaa ctg cag gag aac agc aag atc gcg 1288 D s к I s м А Y N F I L к м I Р F н Ε L 0 Ε N s к I 209 1289 gtc cac gcc aat gcc ttc aga ggc ctc tgc act caa act atc agg gag ata cgg ctc ccc aga aat ggc atc aag gag gtg gcg agt gac 1378 V H A N A F R G L C T O T I R F I R L P R N G I K E V A S D 239 1379 gcc ttc aac ggc acg aag atg tac aga ttg acc cta aaa ggc aac aat ctg ctt act cgc atc agt ccc gac gcc ttt gtg gat tcc agt 1468 A F N G T K M L K G N N L L T R I S P DAF V D S s 269 1469 gac ttg gtg gca ctg gac atc tcc ctg act gcc ctc agc tcc ctg ccg gac tcc atc ctc ggt gga ctc cag agg ctg agc gca gag tct 1558 D L V A L D I S L T A L S S L P D S I L G G L O R L S A E S 299 1559 gcc ctc caa ctg aaa aag ctt ccc aat ctg cag ctc ttc acc aac ctg aac cac gcc agg ctg acg tac ccg tcc cac tgc tgc gcc ttc 1648 ALQLKKLPNLQLFTNLNHARLTYPSHCCAF 329 1649 cag aac gca cac agg aac agg ttg aag tgg aac ccc ctg tgc tcg cac ccc gaa gct ctg gac cac acc aac ttc ttc aga gac tac tgc 1738 A H R N R L K W N P L C S H P EALDHTNFFRDY с 359 O N 1739 cac aac tcc acg tcc atc acc tgc agg acg atg cca gat gag ttc aac ccc tgt gag gac gtc atg tcc acc gtc ttc ctg agg gtc ctc 1828 STSITCSTMPDEFNPCEDVMSTVFL R V L 389 ΗŅ 1829 atc teg atc atc tct atc ctc gcg ctg ctg ggg aac agc ctg gtt ctc ctt ggg tta tta ggc aac ccc tcc aaa ctg acc gtt cct cgt 1918 LG ALLGNSLV G N P S K L т V Р R W s I L L L ι 419 I I Ι 1919 tta gcc gct cca aac tga ctg gag tcc atc gtg tgt ggc tgt tac ctc atg tgt cac ttg gcc ttt gcc gac ctc tgc atg ggg atc tac 2008 TMII LAAPNPLESIV c G С Y L М С HLA F Α D L CMGIY 449 2009 ctg atc gtc ata gcc agc gta gac gtg ctc acc cgc ggc cga tat tac aac tac gcc atg gac tgg cag aag ggc ctg ggc tgc ccc tcc 2098 TMIII YAMDWQKGLG L I V I A S V D V L T R G R Y Y N C P s 479 2099 gcg ggc ttc att acg gtg ttt gcc agc gag ctg tcg gtg ttc aca tta aca gcg atc acc ctg gag cgc tgg cac acc atc acg tac gct 2188 A G F I T V F A S E L S V F T L T A I T L E R W H T I T Y A 509 2189 ctg cgg ctg gac cgc aag atc cgc ctg aga cac gcg tgt atc gtc atg acg gcc ggc tgg atc ttc tca tct ctt gcc gcg ttg ctg ccc 2278 LDRKIRLR LR HACIVMTAGWIFSSLAALLP 539 2279 aca ctc ggg gtc agc agc tac agc aag gtg agt atc tgc ctg ccc atg gac gtg gag tta ctg gag ttc ctag gtc ttt gtt gtg tcc ctg 2368 TLGVSSYSKVSICLPHDVELLES F s L 569 2369 ctc ctc aac atc ctg gcc ttc ttc tgc gtg tgt ggc tgt tac ctc atg tgt ggc tgt tac ctc atg tgt ggc tgt tac ctc atg tgt ggc tgt tac ctc agc atc 2458 LLLNILAFFCVCGCYLNCGCYLNCGCYLSI 599 2459 tat ctg act gtc cgc aac ccc tcg tcg gcg ccg gcc cac gcc gac act cgc gtg gcc cag cgc atg gcc atc ctc atc ttc act gac ttc 2548 Y L T V R N P S S A P A H A D T R V A Q R H A I L I F T D F 629 2549 atc tgc gtg gcc ccc atc tcc ttt ttt gcc atc tcc gcc gcc ctc aag cac cct ctc atc acc gtg tca gac tcc aaa ctg ctg gtg c2638 I C V A P I S F F A I S A A L K H P L I T V S D S K L L L v 659 2639 ttc ttt tac ccg atc aac tcg tgc gcc aac ccc ttc atg tac gcc ttc ttc aac cgc tcc ttc agg cgg gac ttc ttc ctc ctt gcg gcg 2728 F F Y P I N S C A N P F M Y A F F N R S F R R D F F L LAA 689 2729 cgc ttc ggc ctg ttc aag gcc cag gcg cag att tac aag acg gag agt tgt cct gtc agt agc cgg gga aaa caa aga gca gtc acg 2818 ITRTNGEQKQPVAF 🏞 733 2914 aacatcatgt tatagttitt tgtittitta atgcatcica aatatgtigc caatatcett agecggaaaa teageeget actettaga etcaacatgt geaceacaa 3023 3024 aattetaca tgggacaegt etgtegeaaa aceatgggac catgggta catteatet tagtacaata geagtaaga atateaga acaaetgea gaaaaaate 3133 3134 tatgteaaa tgetgteeta tataatteet tgtitgtitt ggettgatg gagtgeeag tgggtggggt ttatgeata taegacata eggtagatt caabaagtgg 3243 3244 taacteettg eagecaact ttattitett ttgettagge agetgtitag ategaatge atteetaga ateatataa taeaggteat taggeagaa 3354 cattlagtig agtacaata attaeggga eccatgggtt taggettegg teegggggt atteetage acattatae taeaggtet tigteetaa 3364 cattaget gatacaata attaeggga eccatgggtt taggetgggg teeetaga gategeage geeseatte eggaagatet ettittig agteecaaca getgetgggg teeetaga 3263 3574 eggeteegg eccatet gateggg geesegg teeggggga ecceggaa eccaggta ageeggetti gttteetate agaaggeta geegggtt aggetgggg 3584 tageeeggt eccegga egeteggt taggetggga ecceggaa aceateggt getteetagt geegget ageegget ageegget ageegget 373 3574 egtgeteegg eccetaegg teetatgga eccegggt taggetggga aegegetti gttteetae tgaagegte geggggt aaaaateea geegegeta 3684 ttgeegegegt teetaggt tagetgtaga acgegget ageegget 373 3794 <u>aaaaacaatg</u> agaacaatg agaacaaag aaaaaaaaa a 3824 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 sus* 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 extragonadal 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 GG-FSHR M-FSHR H-FSHR Consensus	MASLTVLLMI VATTASGPGSENDI KAG. VETSLAKPTTLSCCRLRAGVTEI BSN SSDTRCLEI RCUGLRI POGAVSYLKFLKI LVI VENDM. MVTANVTTVUTSCLALLITNE (VINTAASAPDTNA. GDEATI GKQSLLSSNHLSFGVTEI LFN SSSTFTLVC;ELQVI VI RQVUSHLULTTLI ILENRL MMI MI VI MLMI VM KAAAASAPOPENDI KPGATNGLAKRTLHFCYGLOFGVTEI SSN SSDTQCLEVQGCI KVI PGGALTSLCHLRKLI I SKNDML MMMMMMMMVRSTVLMI VVNNRSA. AETSLTI RIYNPCERL. TEI SSN SSNTCCLEVQGCI KVI PGGALTSLCHLRKLI I SKNDML MMMMMMMMVRSTVLMI VVNNRSA. AETSLTI RIYNPCERL. TEI SSN SSNTCCLEVQGCI KVI PGGALTSLCHLRKLI I LENRML MMRTILMI MLNVVNGAI AS VPGSEVDSEAGFEA. SLAKQTMNSYRLRVVA. TEI PSN SSNTCL EVVQCI RIVPSGAVTSLCHLRKLI I LENRML MRTIL IS MLVHTGNNFL GSVACLASGTTRSFLCLGSKNHQM HH PI NITYVEI KLIQI I FPYRAASSLHD. MLRYI LS GSI ANTEDTLA. ASGFCAFNGSTRSFI CLGNKVHEI PRN PINTTVEI KLIQI SV RRAALSELHE. MVLSMLCFI LGSI ANTEDTLA. ASGFCAFNGSTRSFI CLGNKVHEI MRR PINTTVEI KLIQI SV RRAALSELHE. NPLI FLCLLFSLSPCF. GCHAV. CQCLGRSFVCGESKNEHVINN PRNATELRFVL KKIN I PKGAFAGFCD. MALLLVSLLAFLGSGS. GCHHWLCHCSNRVFLCQDSKVTEI PDD PRNATE RFVL KKIN I PKGAFSGFGD. MALLLVSLLAFLSGS GCHHRI CHCSNRVFLCQDSKVTEI PDD PRNATE RFVL KKIN I PKGAFSGFGD. MALLLVSLLAFLSGS GCHHRI CHCSNRVFLCQDSKVTEI PDD PRNATE RFVL KKIN I PKGAFSGFGD. MALLLVSLLAFLSISS GCHHRI CHCSNRVFLCQSSKVTEI PDD PRNATE RFVL KKIN I PKGAFSGFGD. MALLLVSLLAFLSGS GCHHRI CHCSNRVFLCQSSKVTEI PDD PRNATE RFVL KKRU FLKGAFSGFGD. MALLLVSLLAFLSGS GCHHRI CHCSNRVFLCQSSKVTEI PDD PRNATE RFVL KKRU FLKGAFSGFGD. MALLLVSLLAFLSGS GCHHRI CHCSNRVFLCQSSKVTEI PDD PRNATE RFVL KKRU FLKGAFSGFGD. MALLLVSLLAFLSGS FLKKGAFSGFGD. MALLVSLLAFLSGS FLKKKFVLGAFSGFGD. MALLVSLLAFLSGS FLKKFVLGAFSGFGD. MALLVSLAFLSGS FLKKGAFSGFGD. MALLVSLAFLSGSS FLKKFVLGAFSGFGD. MALLVSLAFLSGSS FLKKGAFSGFGD. MALLVSLAFLSGSS FLKKFVLGAFSGFGD. MALLVSLAFLSGSS FLKKFVLGAFSGFG	94 102 98 88 96 73 78 70 71 71 71
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SM-FSHR CS-FSHR EC-FSHR HH-FSHR SS-FSHR CG-FSHR Z-FSHR XL-FSHR M-FSHR H-FSHR H-FSHR Consensus	SKI ARVHA ARRGICT CTI TE IL UTRNGI KEVASDAFNGT KVYR. TÜKGNULTRI SPDAPUSSDLVALD SE TALSSEDDSI GGG (RESAES ALC) KKEPN GL TN SHI NRI PAARRGICT CTI TE IL TRNGI KEVASDAFNGT KI LRI YURCNKOLTHI HPKAPUSSDLVALD SE TALSSEDDSI GGG (RESAES ALC) KKEPN GL TN SHI NRI PAARRGICT CTI DE IR TIRNGI KEVASDAFNGT KI LRI YURCNKOLTHI HPKAPUSSSDLVLD SE TALSSEDDSI GGG (REFASAFH KEEPP) GL TT NH ARVPSARRGICT CTI DE IR TIRNGI KEVASDAFNGT KNHRI SURGN (QU THI SPDAPUSSSDLVLD SE TALSSEDDSI GGG (REFASAFH KEEPP) GL TT NH ARVPSARRGICT CTI DE IR TIRNGI KEVASDAFNGT KNHRI SURGN (QU THI SPDAPUSSSELVULD SE TALSSEDDSI GGG (REFASAFH KEEPP) GL TT NH ARVPSARRGICT CTI DE IR TIRNGI KEVASDAFNGT KNHRI SURGN (QU THI SPDAPUSSSELVULD SE TALSSEDDSI GGG (REFASAFH KEEPP) GL TT NH ARVPSARRGICT CTI SE IR INFROM KEVASDAFNGT KNHRI SURGN (QU THI SPDAPUSSSELVULD SE TALSSEDDSI GGG (REFASAFH KEEPP) GL TT NH EVI HSAFAGLTSGTI TELRI TRNGI KEVASDAFNGT KNHRI SURGN (QU THI SPDAPUSSSELVULD SE TALSSEDDSI GGG (REFI ARSTECK KEPS) HL TK MH EVI HSAFAGLTSGTI TELRI TRNGI KEVASDAFNGT KNHRI SURGN (QU THI SPDAFK) SSEDVLD SE TALSSEDDSI GGG (REFI ARSTECK KEPS) HL TK NH EVI HSAFAGLTSGTI TELRI TRNGI KEVASDAFNOT KNEKLFING QU THI NTNAFGRSSGLVULD SE TALSSEDDSI GGG (REFI ARSTECK KEPS) HL TK NH EVI SAFAGLTSGTI TELRI TRNGI KEVASDAFNOT KNEKLFING QU THI HSYARKGAEOPVLD SRI ANTI TELSEN KKURA SAVIN KKP AL HE EVI NH EVI SAFAGLTSGTI TELRI TRNGI EL (NAFAFNOT KNEKLFING QU THI HSYARKGAEOPVLD SRI SSEDSKEKKKIKAKASAVIN KKP AL HE EFE NH RI I ERNSFOGLSESVI. LUN KNGI EEI (NAFAFNOT KNEKLFING SKELPEKNECAAI OPVLD SRI SSEDSKEKKIKKIKAKASAVIN KKP AL HE ECZE I HI RTI ERNTSKOGLSESVI. LUN NKNGI GEI HOAFNOT KLELLE SKERLARKEPEKNECAAI OPVLD SRI SFEPSHEKKIKKIKKASAVIN KKP DVNKERS I NH I I ARSSENGLSFESVI. LUN NKNGI GEI HNAFNOT KLELEN SEN NNEELEPENDEHEND SKERSVELSER KEKKIKKIKKIKKINKKIPON KKERS I NH I I ARSSENGLSFESVI. LUN NKNGI GEI HNAFNOT KLELE NI SEN NNEELEPENDEHENDEN SRI SKERSVELSER TIK KKERSVELSESVI I NH I I ERNSSENGLSFESVI. LUN NKNGI GEI HNAFNOT CLEAVINGSEN NNEELEPENDEHENDENDEN SREVENSKERSVERKKIKKKIKKIN KKERSTINK KKEPT KKERSVE	314 309 305 295 303 267 271 262 263 263 263
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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 rhodopsinlike 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. FSHR Acanthopagrus Accession number: 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 mvkiss (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 187



Fig. 5 Quantitative analyses of FSHR mRNA expression in ovarian developmental stages of turbot. Values represent the mean \pm 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 \pm 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 PCRdetection of FSHR in transfected HEK293T cells. GFP-labeled HEK293T cells are denoted by arrows. Trans transfect; CTL control

A





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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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